BDNF AND SUMO PATHWAY MODULATE GEPHYRIN SCAFFOLD TO DOWNREGULATE GABAERGIC TRANSMISSION AFTER ISCHEMIA

Dissertation

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Zusammenfassung

$GABA_A$ Rezeptoren ($GABA_AR$) sind für die schnelle hemmende Neurotransmission im ZNS verantwortlich und synchronisieren die Aktivität von neuronalen Netzen. Die Verfügbarkeit dieser Rezeptoren an Synapsen wird reguliert durch die postsynaptische Dichte (PSD), die das Gerüstprotein Gephyrin enthält. Gephyrin ist ein multifunktionelles Protein, das auch ein hexagonales Gerüst unter den Rezeptoren bildet und dadurch Veränderungen der Plastizität GABAerger Synapsen vereinfacht. Post-translationelle Modifizierung von Gephyrin durch Phosphorylierung, Acetylierung und SUMOylierung spielen eine essentielle Rolle für die Eigenschaften und Stabilität des Gerüsts. In dieser Studie zeigen wir, dass SUMOylierung und Phosphorylierung von Gephyrin verbunden sind und dass SUMOylierung ein wichtiger Faktor ist für die Gerüstbildung von Gephyrin. Wir identifizieren den Wachstumsfaktor Brain Derived Neurotrophic Factor (BDNF) als den vorgeschalteten Modulator der E3 Ligase PIAS-3, die ihrerseits die SUMOylierung von Gephyrin reguliert. Diese Wirkung von BDNF entfaltet sich durch die subzelluläre Lokalisierung und durch funktionelle Änderungen von spezifischen Proteinen des SUMO Signalwegs, im Speziellen PIAS-3. Im Folgenden decken wir zwei unabhängige Mechanismen auf, über die PIAS-3 die Gerüstbildung von Gephyrin moduliert. Ein Mechanismus benötigt die SUMO E3 Ligase-Funktion von PIAS-3; ein zweiter Mechanismus beinhaltet die C-terminale Sequenz und ist abhängig von der ERK1/2 Kinase. Interessanterweise sind SUMO-defiziente Gephyrin-Mutanten nicht empfindlich gegenüber PIAS-3 und dem BDNF Signalweg.

Des Weiteren bauen wir auf Beobachtungen in organotypischen Schnittkulturen auf, kombiniert mit einem Oxygen Glucose Deprivation (OGD) *in vitro* Modell, in dem wir die morphologische und funktionelle Bedeutung des BDNF Signalwegs für den Verlust des Gephyringerüsts nachweisen. Wir zeigen dass die Aktivierung von TrkB PIAS-3 rekrutiert, was zur Herabregulierung von Gephyrin und $GABA_AR$ in der CA1 Region des Hippocampus führt. OGD-induzierte Herabregulierung von Gephyrin kann durch die transgene Expression der SUMO-defizienten Mutationen K148R/K724R in CA1-Neuronen verhindert werden.

Zusammenfassend decken wir einen neuen zellulären Mechanismus auf, der Gephyrin und die Integrität von GABAergen Synapsen unter Ischämie und ähnlichen neurologischen Erkrankungen reguliert.

Abstract

$GABA_A$Rs mediate fast inhibitory neurotransmission in the CNS to control and synchronize network activity. The availability of the receptors at synaptic locations is tightly regulated by the postsynaptic density (PSD) containing the main scaffolding protein gephyrin. Gephyrin is a multifunctional protein that also forms a lattice underneath of the receptors to facilitate structural and functional plasticity of GABAergic synapses. Gephyrin post-translational modifications via phosphorylation, acetylation and SUMOylation play an essential role in determining its scaffolding properties and also scaffold stability. In this study, we demonstrate that SUMOylation and phosphorylation of gephyrin are coupled, and that SUMOylation is an important determinant for gephyrin scaffolding. We identify brain derived neurotrophic factor (BDNF) as an upstream modulator of the E3 ligase PIAS-3, in turn regulating gephyrin SUMOylation. BDNF signaling affects the subcellular localization and induces functional changes of specific proteins of the SUMO pathway, especially PIAS-3. Subsequently, we uncover two independent mechanisms by which PIAS-3 can modulate gephyrin scaffolding. One mechanism, requiring the SUMO E3 ligase function of PIAS-3; and the second mechanism involving the C-terminus sequence in ERK1/2 kinase-dependent manner. Interestingly, gephyrin SUMO defective mutants are insensitive to BDNF signalling.

We further build on our observations by using organotypic slice cultures as an *in vitro* model of oxygen glucose deprivation (OGD), and demonstrate morphological and functional implications of BDNF signaling for gephyrin scaffold loss. We show that TrkB activation results in PIAS-3 recruitment to transiently downregulate gephyrin and $GABA_A$Rs in hippocampal CA1 area. OGD-induced downregulation of gephyrin can be blocked via the transgenic expression of SUMO defective mutations K148R/K724R in CA1 neurons. Taken together, we uncover a novel cellular mechanism regulating gephyrin and GABAergic synapse integrity in brain pathology like ischemia.

I/ General Introduction

The majority of inhibitory neurotransmission is mediated by γ-aminobutyric acid (GABA) and the regulation of the neurotransmitter release is crucial for the maintenance of GABAergic function. GABA is present abundantly in the central nervous system (CNS) and is necessary for maintaining the excitation/ inhibition balance. Gaining knowledge about assembly, maintenance and regulation of GABAergic synapses in the CNS has a crucial relevance for understanding the pathophysiology of multiple neurological and neurodevelopmental and psychiatric diseases. Altered GABAergic transmission has been related to multiple psychiatric disorders such as anxiety, mood disorders [1], schizophrenia [2] in addition to epilepsy [3] and neurodevelopment disorders [4]. In addition, increased inhibitory transmission is associated with Rett, Down syndromes [5] and sedation [6]. Modulation of GABAergic transmission is also clinically relevant, as $GABA_A$ receptors ($GABA_ARs$) are the target of allosteric modulators, such as benzodiazepines (BZs). BZ-mediated enhancement of GABAergic inhibition is a widely used prescription for the treatment of anxiety and insomnia.

Inhibitory synapses are symmetric synapses based on the thickness of the postsynaptic membrane and located primarily at the dendritic shafts [7]. This clear evidence of synaptic contact has been identified for the first time by electron microscopy then by immunochemistry [8]. Electron microscopy allows distinguishing inhibitory neurons from excitatory neurons by the morphology of their synapses. Interneurons possess symmetric electron dense regions whereas excitatory neurons have asymmetric electron dense regions due to the presence of a higher density of macromolecular complexes at the postsynaptic site [7].

Interneurons release GABA from the presynaptic terminals to evoke activation of the postsynaptic $GABA_ARs$. The large cellular varieties of GABAergic interneurons confer them to be classified in 21 classes in the hippocampus. Importantly, they have an essential role in innervating the neighbouring principal cells, also called excitatory pyramidal neurons, in addition to local GABAergic interneurons. Through this innervation, GABAergic interneurons control pyramidal neurons firing pattern thereby synchronising their activity. This process is part of the

regulation of a general network activity by keeping a balance between excitation and inhibition [9].

Changes in neuronal activity lead to changes in synapse gain and functions corresponding to the definition of synaptic plasticity. Two forms of synaptic plasticity have been characterized, local or global. Local plasticity changes, following the Hebbian theory, are described as changes in synaptic strength or number of the presynaptic neurons inducing adaptation at the postsynaptic neurons within a short period of time. However, global changes are long lasting changes and are essential for long-term stability and function of neuronal networks. Thereby, maintain the balance between excitation/inhibition. Interestingly, this homeostatic plasticity allows, in the meantime, local changes at a level of a synapse. It is highly postulated that both type of synaptic plasticity would depend on diverse signaling cascades converging onto the main scaffolding protein at postsynaptic densities (PSD). Therefore, a large variety of studies had been conducted for investigating the importance of gephyrin scaffolding regulation at inhibitory PSDs.

The aim of my thesis is to characterize a novel post-translational modification on gephyrin that modulates its scaffolding properties, leading to functional changes at GABAergic synapses in physiological and pathology.

1. Overview of GABAergic neurotransmission

The rich diversity of interneurons provides inhibition and regulates spatio-temporally the pyramidal cells activity. According to the interneuron cell type, the innervations can be at the level of the soma, at the axon initial segment and at distinct dendritic domains of the pyramidal cells. The classification of the GABAergic interneurons has been made according to their firing pattern and molecular expression profiles [9]. In cerebral cortex, Parvalbumin (PV) interneurons preferentially innervate the somatic compartment of principal cells. Similarly, the axonal initial segment is innervated by the axo-axonic cells, the perisomatic region by basket cells, and the distal and oblique dendrites are innervated by bistratified cells. The apical dendrites located at the *stratum lacunosum-moleculare* (S.LM) of pyramidal cells are innervated by the O-LM cells. Interestingly, GABAergic interneurons contribute temporally to the network activity through

their axonal target specificity and intrinsic properties. They contribute in synchronising pyramidal cells activity by controlling their firing rate and spike timing [10]. Furthermore, by synchronising their activity they also synchronise the general neuronal networks to generate oscillations, in high frequency range, involved in high cognitive functions of the brain such as sleep homeostasis and consciousness.

GABA is synthesized from glutamate by decarboxylation mediated by glutamic acid decarboxylase (GAD). Then GABA is loaded into synaptic vesicles by the vesicular GABA transporter (vGAT) [11]. Upon action potential firing, GABA is released by exocytosis into the synaptic cleft where it can binds to pre- or postsynaptic GABA receptors, followed by reuptake by GABA transporters found pre-synaptically (GAT1) or on the neighbouring glial cell (GAT3). The glial cells metabolise GABA back to glutamate and glutamine, which are then transferred to neurons.

The presynaptic release GABA in the synaptic cleft can act either on the ionotropic $GABA_A$Rs or on the metabotropic $GABA_B$ receptors. $GABA_A$Rs belong to the Cys-loop ligand-gated ion channel family and are ionotropic channels permeable to chloride (and bicarbonate) [12]. They are responsible for most of the fast inhibitory neurotransmission in the CNS and mediate two type of inhibitory transmission according to their localization relative to the postsynaptic density [13, 14]. Synaptic $GABA_A$Rs mediate direct rapid phasic conductance in response to GABA released in the synaptic cleft. Extrasynaptic receptors mediate low persistent tonic conductance in response to ambient concentration of GABA [14, 15]. The function of extrasynaptic receptors is still an emerging concept compared to our current understanding of the synaptic $GABA_A$R regulation. Both types of GABAergic inhibitions are important for brain function and their dysfunction are implicated in neuronal disorders and diseases [6]. $GABA_A$Rs are widespread throughout the CNS and each $GABA_A$R-subunit is expressed in specific brain regions [16, 17] which implicate them in the majority, if it is not all, physiological functions. Our current understanding of the cellular processes that facilitate $GABA_A$Rs function is limited in comparison to glutamatergic synapse regulation. A multitude of interacting proteins has emerged since the past decade helping understanding the receptor trafficking, clustering and their stability at the postsynaptic membrane. Interestingly various interacting proteins are implicated in the

membrane insertion and removal which tightly regulate the receptor number at inhibitory synapses [15].

Trapping the receptors at the postsynapse is necessary for efficient neurotransmission and involved downstream signaling concentrated at the level of GABAergic PSD. $GABA_A$Rs binding partners contribute to the organization of the inhibitory PSD [8]. Among the various $GABA_A$Rs binding proteins are, cell adhesion molecules like Neuroligins (NLs), various cytoskeletal associated proteins, a RhoGEF collybistin (CB) and a scaffolding protein gephyrin [18]. Cooperativity between these molecules is necessary for the maintenance of inhibitory postsynapse [6, 8]. Furthermore, NL2 and CB are important regulators of gephyrin scaffold and in turn $GABA_A$Rs clusters at postsynaptic sites [19]. These $GABA_A$Rs interacting molecules have been associated with different neurological disorders such as epilepsy, schizophrenia and autism [20, 21].

Regulation of $GABA_A$Rs is not only dependent on receptor composition, interacting protein partners, but is influenced by diverse PTMs. In general, both direct and indirect modulations of $GABA_A$Rs contribute to the dynamic modulation of synaptic inhibition [8].

2. Molecular organization of $GABA_A$ receptors

The molecular heterogeneity of $GABA_A$Rs represents a key element in understanding their functions and the regulation of inhibitory neurotransmission in the CNS.

$GABA_A$Rs are pentameric receptors assembled from 19 different subunits genes grouped into seven subunit classes; α(1-6), β(1-3), γ(1-3), δ, ε, θ, π and ρ(1-3), which offer an extensive heterogeneity in their composition [22-24]. $GABA_A$Rs are composed of 5 subunits surrounding a central pore that form the ion channel through the membrane. Each subunit is formed of a large extracellular N-terminus, four hydrophobic transmembrane helices (TM1-4) and a large intracellular loop located between transmembrane 3 and 4. The N-terminus contains the information for subunit assembly and the intracellular domain mediates the interaction with

cytoplasmic proteins [25, 26]. The transmembrane TM2 forms the channel pore and is involved in ion selectivity [27] (Fig. 1a). Despite the numerous combination possibilities provided by the various $GABA_A$Rs subunits and the expression of those subunits in specific brain region, the existing receptors are refined to ~30 different subtypes. $GABA_A$Rs are predominantly formed with 2 copies of α, 2 copies of β and 1 copy of either γ or δ subunits [25, 28, 29] having homologous sequences important for the assembly process [30] (Fig. 1b). The subunit composition influences the feature of the receptors such as their neuronal localisation. $GABA_A$Rs can either be found at the postsynaptic site or at the extrasynaptic sites. The synaptic receptors are containing α1, α2, α3, β and γ2 whereas at the extrasynaptic sites receptors contain α4, α5, or α6 subunits (Fig. 3) [20, 31]. Interestingly, the synaptic receptors need GABA at mM range [32] whereas the extrasynaptic receptors need GABA at μM level to be activated [33]. At a cellular level, no neuronal population has been observed with only the expression of either extrasynaptic or synaptic receptors.

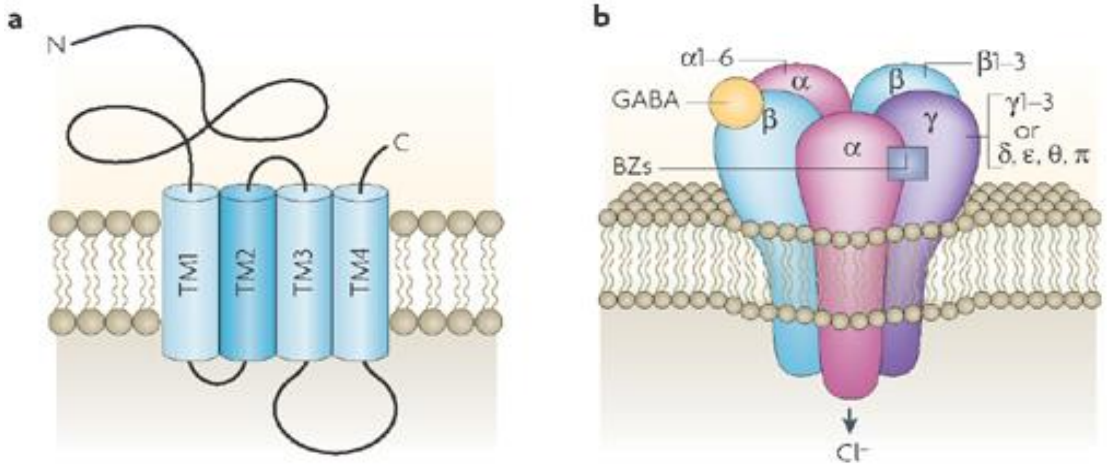

Figure 1: $GABA_A$Rs structure (from Jacob et al. 2008).

a/ $GABA_A$R subunit structure composed of an extracellular N-terminal domain, 4 hydrophobic transmembrane helices (TM1-4) and a large intracellular loop located between transmembrane 3 and 4. **b**/ Hetero-pentameric assembly of $GABA_A$Rs subunits forming a chloride permeable channel formed of 2α, 2β and1γ. 2 GABA binding site are at the interface between α and β and benzodiazepines (BZs) at the interface between α and γ subunits. BZs are allosteric modulator of GABAergic transmission [34].

The receptors stabilization at synaptic sites is partly dependent upon γ2 subunit [31, 35]. However, some receptors containing γ2 subunit are also localised at the extrasynaptic sites and indeed recruited by lateral diffusion for then be at the synaptic site [36].

Therefore, γ2 subunit being abundantly expressed in the brain play a crucial role for $GABA_A$Rs clustering [37], for its activity [38] and is essential in conferring benzodiazepine sensitivity [13, 39, 40].

The functional significance of $GABA_A$Rs heterogeneity is unclear but it endows them with unique functional and pharmacological properties [12, 20]. Moreover, the expression of each subunit is spatio-temporally expressed throughout the CNS and alterations in $GABA_A$Rs expression is directly linked with developmental and neurological diseases [8]. The functional relevance of each subunit has been studied by targeted gene deletion. The existence of different mice strains lacking a specific $GABA_A$R subunit has allowed the characterization of the physiological relevance of $GABA_A$R-subunit for GABAergic inhibition. Those studies contributed I the amelioration of drug development [12].

α1 subunit is the most abundant α subunit in the brain [24] and its knockout (KO) causes 50% loss of total $GABA_A$Rs. This is similar to the observations made using β2 subunit KO mice. Despite significant loss of $GABA_A$R expression levels, both α1 and β2 KO strains show little behavioural consequence [41]. Interestingly, α1–deficient mice exhibit a compensatory mechanism by increasing the expression levels of certain $GABA_A$Rs subunits such as α3 and α4 accompanied by a disruption of γ2 subunits and gephyrin clustering [42] in addition to a decrease in GABA currents [43].

Differently to α1–KO or β2–KO, mice lacking β3 or γ2 subunits do not survive. Those genetic mutations are neonatal lethal [40]. β3–deficient mice show a decrease in $GABA_A$Rs containing α2/3 surface expression and no compensatory upregulation of the others β subunits [44].

$GABA_A$Rs containing γ2 subunits are the most predominant in the brain and its deletion is lethal [31, 40]. The disruption of its gene didn't show any upregulation in one of the other subunits but induces severe behavioural and sensorimotor dysfunctions [40]. The lethally can be prevented by overexpressing either the long or the short splice isoform of γ2 subunit in the γ2-KO mice. In the

surviving mutants mice, the loss of subunits was reflected by a general loss of $GABA_A$Rs and the scaffolding protein gephyrin expression; which can also be rescued by one of the isoforms overexpression [45]. In α2-KO mice, ones see a similar loss of postsynaptic current and a decrease in gephyrin expression. However, there is no replacement of the missing subunit with α1 or α3 subunit. A specific loss of α1-$GABA_A$Rs, gephyrin and NL2 can be seen in the axon initial segment but not in the perisomatic synapses of the α2 KO mice. These striking effects can be explained by the presence of dystrophin-glycoprotein complex at the perisomatic site, known to be involved in the regulation of $GABA_A$Rs clustering at these sites [24].

It follows from those KO studies that $GABA_A$Rs-containing α1 subunits possess potential role in sedation, anticonvulsant and are involved in addiction and memory functions. However, $GABA_A$Rs-containing α2 or α3 subunits are implicated in sleep and have an anxiolytic and myorelaxation roles. Moreover, α2 has an antidepression action [46].

On the other hand, δ2-KO mice show a down-regulation of α4 subunits associated with an up-regulation of γ2 subunits. Behavior experiment highlights the decreased sensitivity of these KO mice to neurosteroïds. This functional deficit is accompanied with a reduction in tonic inhibition while its consequence remains unclear [47].

The characterization of the $GABA_A$Rs subunit specific KO mice show adaptive change in the subunit composition and compensatory expression changes in the brain leading to the reorganization of GABAergic circuits. Interestingly, the receptor subunit composition is a key determinant for their pharmacological profile [23].

2.1 Pharmacology of $GABA_A$Rs

$GABA_A$Rs are macromolecular complexes regulated by different pharmacological reagents. The opening of the channel is achieved through the binding of two molecules of GABA on sites located between α and β subunits [48]. The receptors can also be activated via a selective agonist called muscimol [49], whereas competitive antagonists include bicuculline, picrotoxine, gabazine or 3-amino-propane sulfonic acid [50]. In contrast, the application of $GABA_A$R antagonist,

gabazine, leads to an increase in the number of receptors at the synapse by slowing down the diffusion of the receptors [51]. The effect of gabazine is only on the synaptic $GABA_A$Rs as the extra-synaptic receptors are insensitive to gabazine [52]. $GABA_A$Rs also represent major sites of action of BZs, barbiturates, neurosteroids, ethanol and some general anaesthetics [13, 49]. They all modulate, in an allosteric manner, the GABA binding therefore modulate the channel opening probability and current [50, 53]. Many of these drugs are involved in the enhancement or reduction of GABAergic neurotransmission, depending on the ligand used, for their clinical actions [54, 55].

BZs bind at the interface between α and γ subunits of $GABA_A$Rs and modulate GABA responses (Fig. 1b). $GABA_A$Rs containing α1, α2, α3 and α5 are highly sensitive to classical BZs, such as diazepam, in contrary to the one containing α4 and α6. α4 and α6 are diazepam-insensitive. This difference could be explained by the presence of a conserved histidine amino acid relevant for BZs sensitivity whereas the other subunits carry an arginine at the equivalent residue position [56]. Interestingly, the disruption of γ2 gene (γ2-KO mice) leads to non-detectable BZs binding, which do not affect the embryonic development [40]. Therefore, γ2 subunits are postnatally necessary for BZs modulation and for normal brain function.

BZs are allosteric modulators used in clinical therapies for their anxiolytics, sedatives, anticonvulsants and muscles relaxant properties. In general, BZs, such Diazepam, Lorazepam or Flunitrazepam are positive allosteric modulators, enhancing GABA responses. To investigate which of the $GABA_A$Rs subunits were responsible of the anti-hyperalgesic action in condition of inflammation, transgenic mice harboring a point mutation (H/R) on the conserved histidine amino-acid in $GABA_A$R α subunit into an arginine was generated. H/R mutation in α1, α2, α3 and α5-$GABA_A$Rs subunits variants shows a loss of specific effect of diazepam in those knock-in mice. The receptors containing point-mutated subunit variants became insensitive to diazepam [57, 58]. Moreover, by these studies it could be determined that the effect of sedation is mainly via α1 subunit, the effect of anxiolytics is via α2 subunit [56, 59] and the effect of myo-relaxation is via α3 subunit [60].

Besides BZs, $GABA_A$Rs are also target of barbiturates, depressant drugs enhancing or mimicking GABA response. Barbiturates are positive modulators prolonging and potentiating the effect of

GABA but at high dosage they can act as an agonist of GABA and directly activate $GABA_A$Rs [61]. They are derived from barbituric acid and have a wide spectrum of effects such as hypnotics, anxiolytics and anticonvulsants. However, because of tolerance induction, they have been medically replaced by BZs.

Among the variety of drugs modulating $GABA_A$Rs are the endogenous neuroactive steroids or neurosteroids [62] synthesised during stress, pregnancy and alcohol intoxication, which act at different levels of a synapse. At the presynapse, neurosteroids modulate vesicular release whereas at the postsynapse they modulate the excitability of the neurons in response to stress via activation of diverse mechanism. Neurosteroids have the ability to activate $GABA_A$Rs by binding to the GABA binding site and/or to potentiate the GABA response by binding $GABA_A$R in a hydrophobic pocket present in α-subunit. Moreover, one of the main targets of the neurosteroids is the extrasynaptic $GABA_A$Rs containing the δ subunit. By occupying the activation and potentiation sites, steroids are able to enhance GABA response, therefore fine tune neuronal inhibition. However, the neurosteroids activity is dependent on residues located at M1 and/or M2 transmembrane domains of α1-subunit [62]. Neurosteroids synthesis is related to sex-differences in the genomic regulation of their own synthesis and degradation [63]. Moreover, neurosteroids can regulate $GABA_A$Rs at the transcriptional level under pathological conditions such as epilepsy, stress, schizophrenia and Alzheimer disease (reviewed in [64, 65]). Rapid declines in circulating neurosteroids are correlated with increased seizures and anxiety susceptibility. Thereby, they represent a potential therapeutic treatment of seizures and diverse psychologic disorders. Interestingly, there is a direct correlation between the concentration of neurosteroids and $GABA_A$Rs gene expression (mRNA levels). The increase in neurosteroids during pregnancy is observed in parallels with a decrease γ2 subunits mRNA levels. In this case, neurosteroids exert a positive allosteric modulation on $GABA_A$Rs surface expression, subsequently, act on GABAergic transmission. This effect is reversed after parturition where the neurosteroids levels are decreased [64]. Neurosteroids play an important role in the modulation of neuronal plasticity related to divers' biological functions such as mood, stress and emotional responses.

Interestingly it has been shown that changes in the level neurosteroids are not associated with changes in the scaffolding molecule gephyrin [66].

2.2 $GABA_A$Rs-trafficking

$GABA_A$R subunits are synthesized and assembled in the endoplasmic reticulum (ER) and matured in the Golgi [67]. During the maturation stage the receptor availability is regulated by endoplasmic reticulum quality control machinery (ERAD) via ubiquitination and degradation [68]. After the initial quality control the receptors traverse the secretory pathway to reach the plasma membrane extrasynaptically, later getting recruited to the postsynaptic sites via lateral diffusion [25] (Fig. 2). Therefore, dynamic regulation of reserve pool of extrasynaptic receptors is an important determinant of phasic synaptic inhibition [36, 69]. Nevertheless, receptor trafficking and accumulation at synaptic sites is controlled by endocytosis and exocytosis which also regulate $GABA_A$Rs synaptic inhibition efficacy (reviewed in [15]).

At the level of the ER, receptor assembly is limited by constitutive ER association degradation (ERAD). Afterwards, maturation of the assembled receptors in the Golgi via post-translational modification (PTM) influences receptor accumulation. For example, $GABA_A$Rs γ2 subunit is subject to palmitoylation in the Golgi apparatus, promoting receptor progression to the secretory vesicles. This PTM is facilitated by the Golgi-specific DHHC zinc finger protein (GODZ) promoting $GABA_A$Rs translocation from the Golgi apparatus to the plasma membrane [70]. The exit of $GABA_A$Rs from the Golgi is facilitated by a number of proteins interacting with the receptors. Amongst these diverse proteins, $GABA_A$R-associated protein (GABARAP) has gained prominence over the past decade. GABARAP interacts with all receptors through their γ subunits and with the microtubules and facilitates $GABA_A$Rs translocation to cell surface [71]. The importance of GABARAP interaction with $GABA_A$Rs for receptor function is still a subject of debate as GABARAP-KO mice show normal cell surface expression of $GABA_A$Rs containing γ2 subunits. In addition, GABARAP has a multitude of interacting partners, which complicates our current understanding of the regulation process [15], and moreover GABARAP is absent at synapses [72].

Endocytosis of $GABA_A$Rs is a critical step that tightly regulates the physiological and pathological adaptations for neuronal excitability. For effective endocytosis, $GABA_A$Rs have to reach the dendritic endocytic zones, where they interact with clathrin-adaptor protein AP2. By

interfering with AP2 binding motif within the intracellular loop of $GABA_AR$-β3 subunit the receptors are stabilized at the plasma membrane. Moreover, phosphorylation of $GABA_AR$-β subunits negatively regulates AP2 interaction, influencing the residency time at the endocytic zones and steady-state synaptic receptor levels [73]. Independent studies using single particle tracking have demonstrated that $GABA_ARs$ are trafficked along the plasma membrane in an activity-dependent mechanism and PTM of specific receptor subunits might play a role in this regulation [74, 75]. The PTM of $GABA_ARs$ could also influence interaction with the main scaffolding molecule gephyrin restraining $GABA_ARs$ mobility at GABAergic postsynaptic sites [76].

Interestingly, activating $GABA_ARs$ by muscimol causes a decreased interaction between $GABA_ARs$ and gephyrin leading to an increase in the lateral diffusion of the receptors at the postsynapse. However, a treatment with the BZ antagonist like flumazenil regulates $GABA_ARs$ trafficking and decreases their surface expression leading to a decrease in inhibitory neurotransmission [77]. Therefore, BZ contribute in stabilisation of the receptors at the synapse [78].

The diffusion coefficient of $GABA_ARs$ is dependent on Ca^{2+} influx and therefore is directly linked to the rapid adaptations in GABAergic transmission. Increase in Ca^{2+} influx, upon neuronal activity, reduces evoked inhibitory postsynaptic potentials (eIPSPs) [79] and mIPSCs, due to the dispersion of synaptic $GABA_ARs$ to the extrasynaptic sites. The increased diffusion coefficient of $GABA_ARs$ involves activation of the Ca^{2+} -sensitive phosphatase calcineurin [74]. Calcineurin reduces the resident time of quantum dot-labeled single $GABA_AR$ molecules via dephosphorylation of the γ2 subunit at Serine 327 [80]. In addition, by increasing extrasynaptic diffusion coefficient, the receptors are subsequently internalised. This process has been correlated with a loss of $GABA_AR$ function after status epilepticus [81]. This observation is contradicted by an earlier study showing calcineurin induced long-term depression of GABAergic inhibition [82].

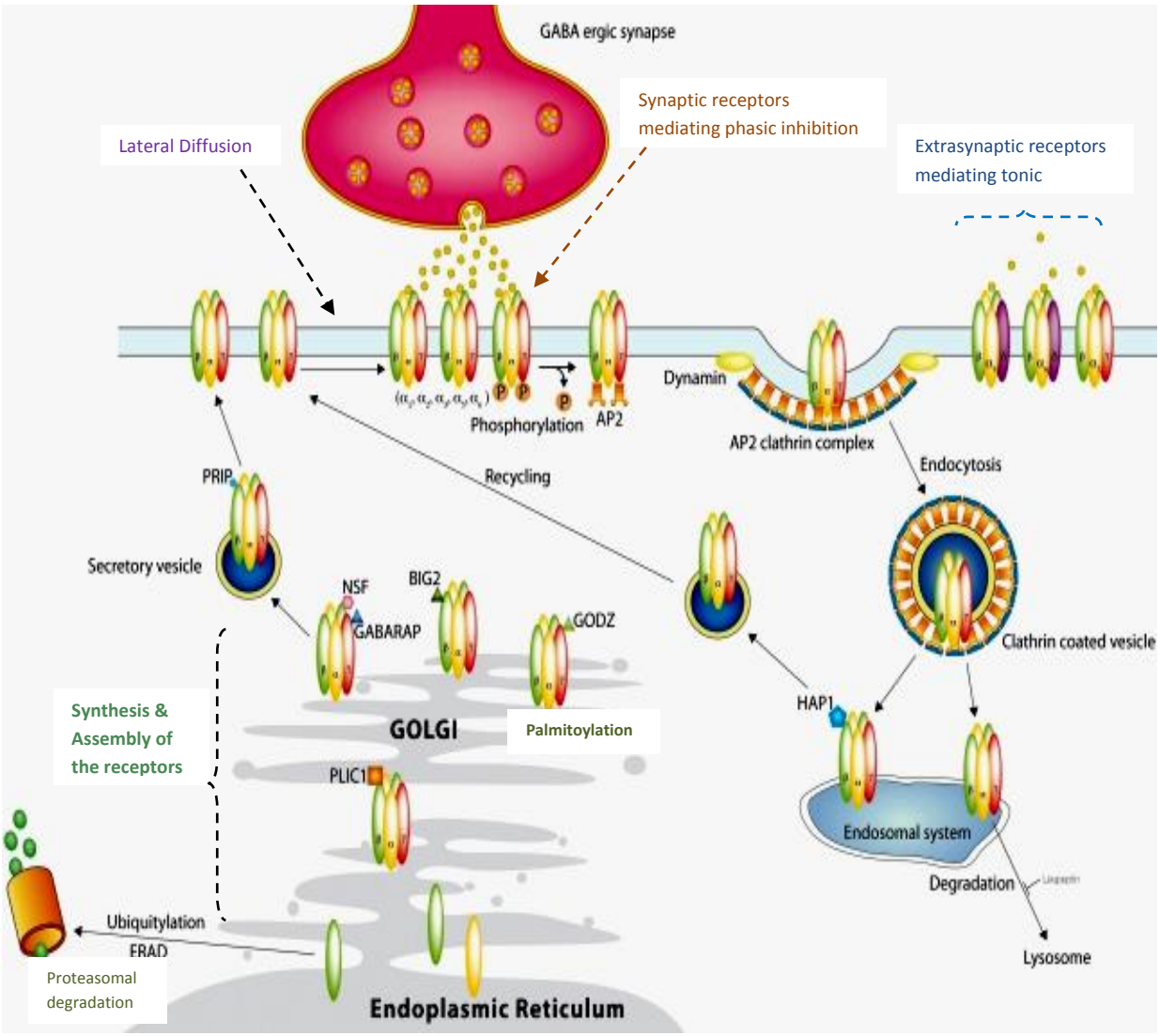

Figure 2: $GABA_A$ receptors trafficking (adapted from Tretter and Moss, 2008).

$GABA_A$Rs are synthesized and assembled in the reticulum endoplasmic. After maturation in the Golgi the receptors, through the secretory path, reaches the plasma membrane. By lateral movement, synaptic receptors reach their destination. Trapping and removal of the receptors are regulated by phosphorylation. AP2 dephosphorylation lead to clathrin endocytosis of the receptors. In the endosomal system, either the receptors are recycled or send to lysosomal degradation [25].

One way to reconcile these regulatory differences could be that short chemical (NMDA) stimulation leads to enhancement of $GABA_A$Rs surface expression and GABAergic transmission. Whereas a strong stimulation (high frequency stimulation of glutamatergic afferents) would depress GABAergic inhibition by endocytosis of the receptors [15].

Endocytosed receptors are either recycled and sent back to the plasma membrane or sorted for lysosomal degradation. Studies exploring the endocytic lysosomal pathway have mainly focused on $GABA_A$R-γ2 subunit containing receptors as they consist of majority of the synaptic receptors. They observed that prior to degradation the γ2 subunit is ubiquitinated on series of lysine residues located within the intracellular domain [83]. Moreover, a decrease in $GABA_A$Rs ubiquitination by increasing the neuronal activity leads to stability of the receptors at the plasma membrane [83, 84]. However, the ubiquitin ligases involved in ubiquitin-mediated $GABA_A$Rs degradation remain unknown.

These studies on $GABA_A$Rs trafficking prove that receptors number at the synapses is determined by local trapping and lateral diffusion [85]. The receptor trafficking modulation requires essentially the participation of different post-translational modifications (PTMs), such as phosphorylation of different $GABA_A$Rs subunits. Moreover, the main scaffolding protein gephyrin, in cooperation with other synaptogenic molecules, contributes in $GABA_A$Rs diffusion properties by trapping the receptors at the postsynapse sites [76]. Thus, dynamic trafficking of the receptors represents prevalent form of GABAergic neuronal plasticity [15].

2.3 $GABA_A$Rs post-translational modifications

$GABA_A$Rs are subjected to post-translational modifications, which have been well established to be key modulators of the receptor number and localization therefore contributing to the heterogeneity in the GABAergic transmission regulation. The main PTMs involved in this tight regulation are phosphorylation and ubiquitination. The best characterized $GABA_A$Rs PTM is phosphorylation [86]. Multiple phosphorylation mechanisms act in concert to modulate the receptors kinetics properties or channel gating, the receptors stability, their cell surface integration and their internalisation [15, 34].

Interestingly, only one site of phosphorylation has been reported at α-subunits to be of high interest. A proline-directed kinase phosphorylation site has been identified at α1-subunits regulating the interaction between $GABA_A$Rs- α1 subunits and gephyrin. Dephosphorylation of the threonine 375 prevents the interaction of the subunit with gephyrin thereby increasing its diffusion at the membrane. This study confirms the importance of gephyrin binding to the receptors for modulating their diffusion properties therefore regulating their accumulation at the postsynapse [87].

β2, β3 and γ2 subunits represent the main phosphorylated subunits and substrates of various kinases, including protein kinase A (PKA), protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) [38, 88]. PKC have been shown to phosphorylate β1 and β3 subunits and PKA to phosphorylate β2 subunits. Phosphorylation by both kinases occurs at the subunits AP2 interacting sites, altering $GABA_A$Rs internalisation and trafficking [89]. PKA-mediated phosphorylation at $GABA_A$Rs β subunits is reverted by protein phosphatase 1α (PP1α). Endocytosis of $GABA_A$Rs β subunits is further regulated by the phospholipase C-related catalytically inactive proteins 1 and 2 (PRIP1/2). PRIP 1/2 act as adaptors for PP1α and PP2A by activating them subsequently; hence, dephosphorylating the $GABA_A$Rs–β subunits at the AP2 interaction site. Thereby, PRIP1/2 facilitates $GABA_A$Rs endocytosis.

Interestingly, CaMKII can phosphorylate at the same sites than PKC or PKA [88] regulating the functional properties and membrane insertion of β2 and β3 subunits at postsynaptic sites. Consequently, phosphorylation by CaMKII enhances $GABA_A$Rs surface expression and GABAergic tonic currents [90]. CaMKII is normally localised at dendritic spines suggesting that CaMKII might translocate from spines to the dendritic shaft. Therefore, these findings highlight the possibility that excitatory transmission on the target neurons increases GABAergic activity.

Phosphorylation of the β3 subunit at S408 and S409 residues enhances $GABA_A$Rs channel activity whereas phosphorylation of γ2 subunits regulates GABAergic tonic and phasic inhibition [91]. The γ2 subunit can be phosphorylated by PKC and CaMKII at two residues, on their cytoplasmic loop, which are important in the regulation of receptor internalisation [88]. The generation of knock-in mice carrying point mutations on two tyrosine sites Y365/Y367 mimicking their dephosphorylation lead to embryonic lethality. Heterozygote mice show sex-

specific increase in tonic-inhibition, as only female are affected, which present an upregulation of the extrasynaptic $GABA_ARs$ containing α4 and δ subunits [91]. Interestingly, the Y365/Y367 tyrosine residues have been shown to be phosphorylated by the tyrosine kinase Src [38].

In addition to all those kinase cited above, the serine/threonine kinase Akt is required for $GABA_ARs$ cell surface activation. Akt phosphorylate a conserved phosphorylation site on $GABA_AR$-β subunits. Phosphorylation of the β2 subunit at S410 or β1–3 subunits at S409 enhances $GABA_ARs$ surface expression and regulates GABAergic synaptic strength [92]. Interestingly, Akt activation leads to inhibition of GSK3β kinase activation. The inhibition of GSK3β activity has been linked to a reduction in gephyrin phosphorylation state contributing indirectly to the enhancement of GABAergic inhibition [93].

Thus, de-phosphorylation increases the lateral mobility of the receptors but without involving endocytosis [80]. However, this process is often subsequently followed by their removal from the surface and thereafter their internalisation [15]. Another negative regulator of $GABA_AR$ clustering at synaptic sites is the phosphatase peptidyl-propyl isomerase NIMA interacting protein1 (Pin-1) [94].

In general, dynamic mechanisms such as PTMs regulate the receptor complexity, stability and internalisation. Thereby, regulate GABAergic transmission efficacy, thus neuronal excitability in the CNS. Consequently, PTMs contribute to the formation and function of GABAergic synapse [70, 95, 96].

2.4 Regulation of GABAergic transmission by BDNF

BDNF regulates the development and plasticity of GABAergic synaptic transmission in the CNS [97-100], in part through the regulation of surface expression of $GABA_ARs$ (Fig. 4) [101, 102].

BDNF is stored in dense core vesicles of excitatory neurons [103] and is synthesized as a precursor named proBDNF. proBDNF is then proteolytically cleaved either inside the cell or after its secretion in the synaptic cleft to form mature BDNF (mBDNF). Independent studies brought evidence that mBDNF is secreted in response to neuronal depolarisation thereby

influencing neuronal activity [104]. mBDNF bound to its high-affinity tropomyosin-related kinase B (TrKB) receptor localised at pre- and post- synaptic sites [105] acting, hence, on excitatory and inhibitory synapses. BDNF/TrKB interaction induces the dimerization of the receptor which thereafter auto-phosphorylate and activates different intracellular downstream signaling cascades (Fig. 3). So far, three signaling transduction pathways have been well characterized: the phosphatidyl-inositol 3 kinase (PI3K), the mitogen-activated protein kinase (MAPK) and the phospholipase Cγ (PLCγ) [106]. PI3K activates Akt which in turn inhibits the glycogen synthase 3β (GSK3β) activity, contributing to neuronal survival. MAPK pathway would lead to the activation of the extracellular signal related kinase (ERK) involved in the activation of several downstream effectors implicated in cell growth and differentiation. In the case of PLCγ, this pathway leads to the activation of inositol-triphosphates (IP3) and thereafter calmodulin kinase (CamK) activity, crucial during synaptic plasticity. Interestingly, all three signaling cascades converge onto the activation of the transcriptional factor cAMP response-element binding protein (CREB), subsequently upregulating gene expression [106] (Fig. 3).

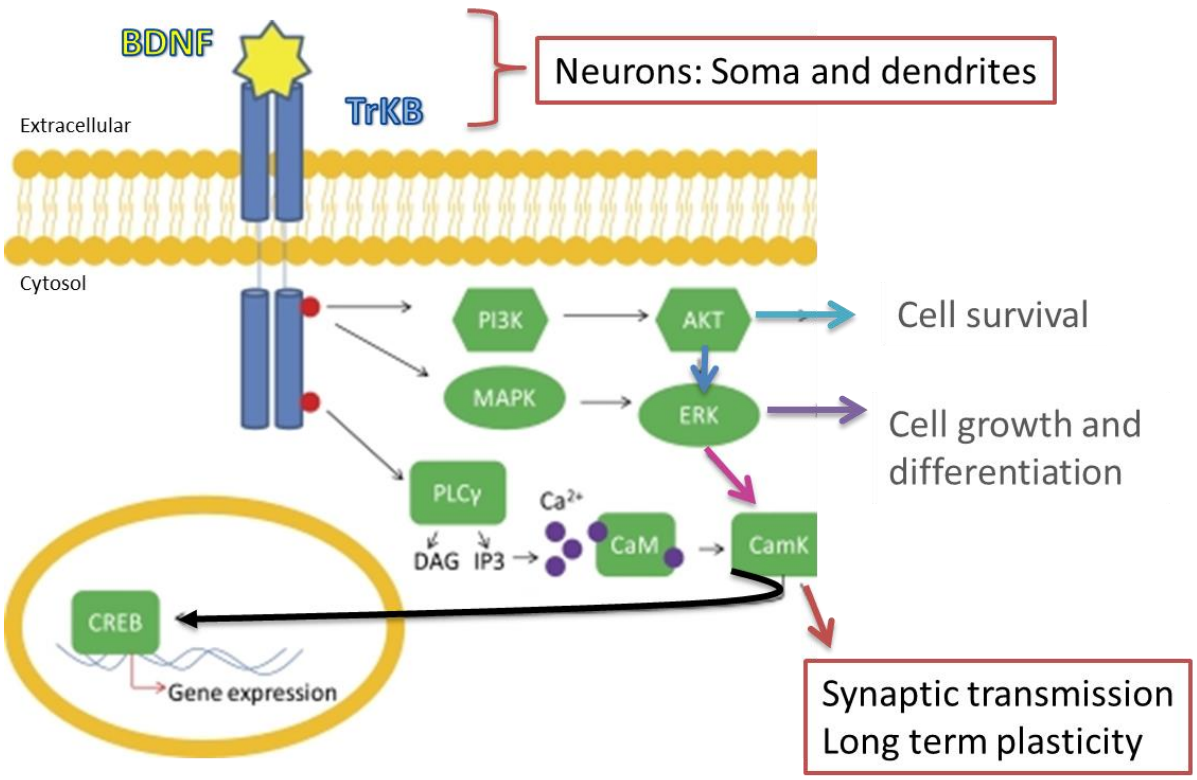

Figure 3: **Overview of BDNF signaling through TrkB receptors (adapted from Autry & Monteggia, 2012)**.

In mature neurons, BDNF has dual opposite effects depending on the duration of its application producing a biphasic effect on GABAergic transmission. Acute and chronic BDNF effects on GABAergic synapses occur via modulation of the phosphorylation state of $GABA_A$Rs.

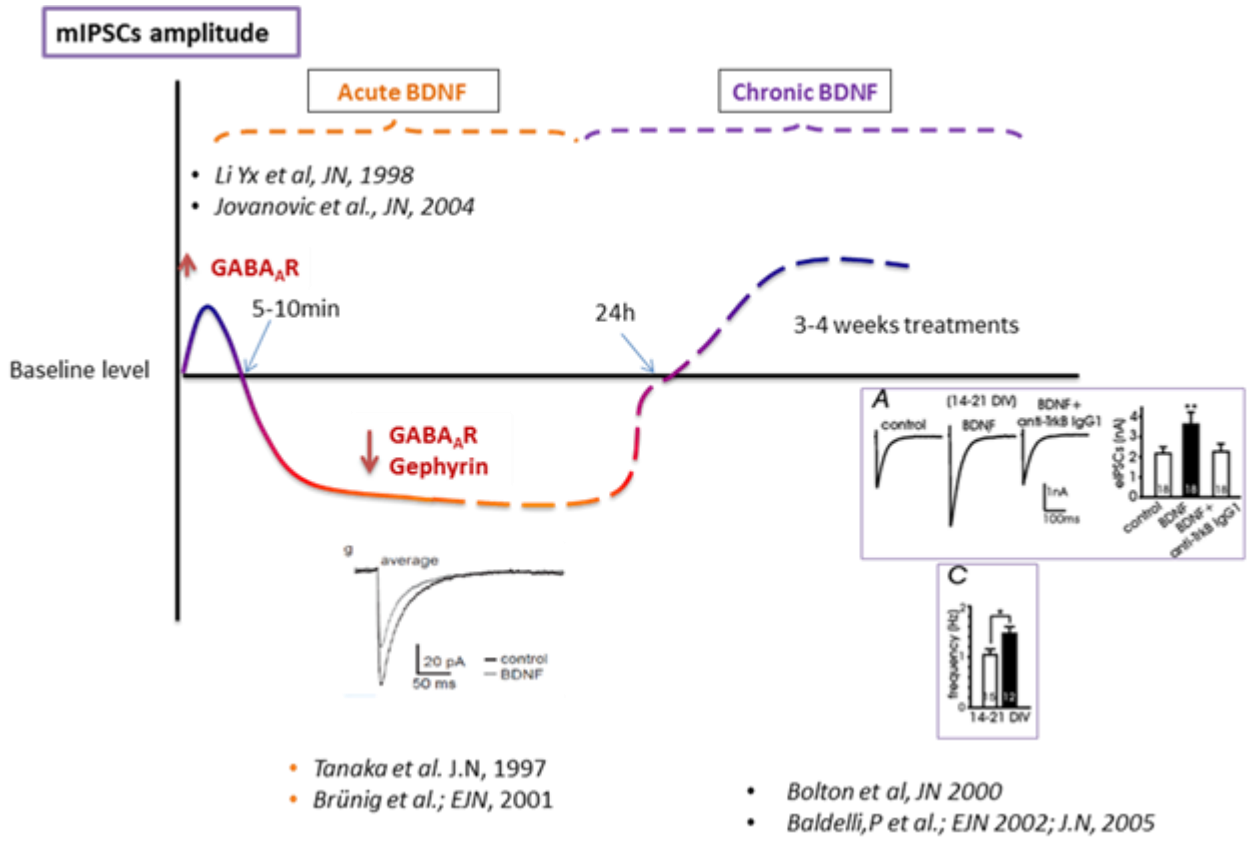

Figure 4: BDNF modulation of GABAergic transmission in hippocampal neurons.

The coloured line represent the variation of GABAergic mIPSCs amplitude trough the time. Following the literature result, it could be observe that BDNF has different effect according to its time application. Are added examples average amplitude and frequency of GABAergic inhibitory postsynaptic currents (mIPSCs, eIPSCs) illustrating BDNF bidirectional modulation. Only acute treatment of BDNF has induced variation in $GABA_A$Rs surface expression.

Acute BDNF exposure weakens GABAergic transmission [107, 108] and $GABA_A$Rs surface expression [101] in hippocampal neuronal cultures. Application of 100ng/mL of BDNF is sufficient to induce significant reduction, within 5min, in mIPSCs in a subset of hippocampal neurons [101]. Unexpectedly, an amplitude increase was observed within the first two min, followed by a decrease (Fig. 4). In a later study, it was also shown that 200ng/mL of BDNF induced a rapid increase in the mIPSCs amplitude of CA1 neurons, followed by long lasting depression within the next 20min [109] (Fig. 4). In both these studies approximately 70% of the cells were shown to express the TrKB receptors. So by the use of the TrKB antagonist: K252a (200nM), they could inhibit the BDNF effect on GABAergic transmission [101, 108]. K252a is a membrane-permeable tyrosine kinase inhibitor [110, 111], which, among others blocks TrKB phosphorylation.

Subsequent studies have shown that BDNF/TrKB signaling involves the activation of different kinases to influence the phosphorylation state of different $GABA_A$R subunits. A short application of BDNF (100ng/mL) induces a reduction of α2, β2, β3 and γ2 subunits immunoreactivity [108]; however, only the β3 subunit shows a reduction in phosphorylation state. PKC phosphorylation of the residues S408/S409 in β3 subunit could enhance the binding of the receptors to PP2A and the phosphatase adaptor PRIP, in turn causing dephosphorylation of the receptors at those sites at a later stage [109]. Therefore, these phosphatases would facilitate clathrin-mediated endocytocis of the receptors. This acute modulation of GABAergic mIPSCs by BDNF involves biphasic modulation of phosphorylation state of $GABA_A$R-β3 subunits at S408/S409 sites. Recent studies also implicate phospholipase C (PLC) in the downstream signaling pathway in addition to PKC and PP2A [112]. In addition, rapid degradation of gephyrin could also contribute towards the rapid internalisation of $GABA_A$Rs in the amygdala [113, 114].

Contrary to acute BDNF application, chronic BDNF application enhances $GABA_A$Rs surface expression and the efficacy of GABAergic inhibition (Fig. 4) [115, 116]. The chronic effect of BDNF at GABAergic synapses is mediated trough the phosphorylation of γ2 subunits at two tyrosine residues 365 and 367 (Y365/7) leading to an increase in the residence time of the receptors at synaptic sites [117]. However, first messengers, downstream of BDNF, that

eventually influence γ2 subunit phosphorylation at remains unidentified. Otherwise, it has been shown, in non BDNF-condition, that phosphorylation of γ2 subunits at Y365/7 residues is mediated by PKC and CaMKII [88]. In response to a long-term alteration of neuronal activity, BDNF can modulate inhibitory synaptic strength[118]. Independent studies have shown that chronic BDNF availability preserves $GABA_AR$ density [115] without affecting the synapse density but increasing their size [116].

3. GABAergic postsynaptic density

3.1 Gephyrin

Our current understanding of the GABAergic PSD is still incomplete. However, the main scaffolding protein gephyrin has been studied in some detail to gain insights into inhibitory synapse assembly and maintenance. Its relevance at GABAergic synapse has gained much from these findings.

Gephyrin is a well conserved multifunctional protein of 93kDa that is essential for Molybdenum cofactor biosynthesis. Within mammals, gephyrin is widely expressed in all tissue types with its diverse alternative splice variants [119]. The deletion of *GPHN* leads to neonatal death [120, 121]. Gephyrin was first identified as a glycine receptor interacting protein [122] then at GABAergic synapses [123, 124]. Its genetic deletion induces the lack of postsynaptic glycine receptors and some $GABA_ARs$ at the synapses, leading to a reduction of respiratory motoneuron survival thereby affecting the respiratory system. Much of the synaptic gephyrin function has been attributed to its self-oligomerizing property [18] (Fig. 5). More importantly gephyrin serves as a reliable GABAergic postsynaptic marker [20].

Gephyrin is composed of two conserved domains: N-terminus G-domain (20kDa) and the C-terminus E-domain (43kDa) connected by a linker domain or central C-domain (18-21kDa) [18, 125]. Gephyrin structure is not clearly defined as the two conserved domains were crystallized but not the C-linker domain. It is well accepted that gephyrin G-domain can form trimers and the

E-domain forms dimers [126], providing the basis for a putative hexagonal sublattice gephyrin organization at GABAergic synapses [127, 128].

The gephyrin C-domain linker is sensitive to proteolytic degradation and contains multiple consensus sites for phosphorylation. In addition, it also carries binding sites for various gephyrin-interacting proteins, like Collybistin (CB), Pin1, Dynein light chains (Dlc) and microtubules [119]. Thus, gephyrin at the PSD may organise the spatial distribution of the receptors and others proteins by forming a microfilament-associated hexagonal protein lattice. The G and E-domains also carry binding sites and possess, as the C-linker domain, post-translational sites important for the regulation of gephyrin oligomerization properties. Moreover, the E-domain of gephyrin is crucial for regulating the clustering function. Mutating or depleting the E-domain prevents the clustering of gephyrin and the formation of α2-$GABA_A$Rs at the plasma membrane. However, over-expressing a specific sequence of the E-domain enhances gephyrin cluster density [125]. The oligomerized forms of gephyrin are found at GABAergic and glycinergic PSDs visualised as distinct punctate structures by immunochemistry [87, 129]. Gephyrin interaction with diverse $GABA_A$Rs subunits such as α1, α2, α3, β2, β3 and γ2 subunits have been reported [25, 72, 87, 130, 131]. Super resolution microscopy-based quantification places gephyrin molecules associated with $GABA_A$R is in a stoichiometry of 1:1 [132]. Further, inhibitory PSDs comprised between 3000-10000 gephyrin molecules and the number of $GABA_A$Rs at the synapse is dependent on gephyrin abundance [132]. Furthermore, by trapping and accumulating the receptors at the synaptic sites, gephyrin does play a role on the surface dynamic of $GABA_A$Rs at the membrane. Deficiency in gephyrin leads to a loss of $GABA_A$Rs surface expression [31, 124], correlating with the concept that changes in the number of gephyrin molecule are mirrored with changes in the receptors number. Interestingly, the γ2 subunit, for which there is still no evidence for direct gephyrin interaction, somehow facilitates gephyrin recruitment and clustering at postsynaptic sites [45].

In addition to the $GABA_A$Rs, gephyrin can interact and be regulated by various synaptogenic molecules such as (CB) [19], neuroligin 2 (NL2) and cytoskeletal associated proteins such as microtubules and profilin 1/2 [133].

Gephyrin is subject to diverse PTM and its relevance is emerging slowly. Some of the intra-cellular signaling cascades converging on to the gephyrin scaffold has been identified and

characterized in literature. Since gephyrin scaffolding and $GABA_ARs$ function are coupled, it is therefore conceivable that gephyrin PTM regulates the efficacy of GABAergic transmission at synaptic sites.

3.2 Gephyrin regulation by RNA splicing

GPHN gene encodes several introns that are alternatively spliced generating different splices isoforms [119]. Expression of specific gephyrin splice cassettes in distinct tissue types has been reported. The splice cassettes in the case of GPHN are as short as 33 amino acids, leading to difficulties in specifically identifying isoform specific functions *in vivo*. Gephyrin variants containing the G2 (formerly C5) cassette exhibit a specific preference for glycinergic synapses as they prevent Glycine receptors to cluster at GABAergic synapses [134]. It has been shown that insertion of G2 cassette produces the dimerization of the G-domain. Similarly, expression of C3 cassette seems to be responsible of E-domain oligomerization [135]. Thus, gephyrin splicing is an additional regulatory step essential for ensuring the proper sorting of the receptors to appropriate synapses. Moreover, it has been noticed that the gephyrin splice variants expression is dependent on the tissue and species [136]. Nevertheless, *in vivo* all gephyrin splice variants contain C2 and C6 splice cassettes.

Temporal lobe epilepsy had been linked to an abnormal splice variant of *GPHN* mRNA lacking exons coding for the G-domain of gephyrin. The presence of those exons is essential for gephyrin/ $GABA_ARs$ clustering and stability [137].

3.3 Gephyrin interacting proteins

Gephyrin has been discovered as the main scaffolding protein at glycinergic synapses and is essential for the clustering of glycine receptors. However, at GABAergic synapses gephyrin is in part dispensable for the clustering of $GABA_ARs$ [138, 139]. Gephyrin interacts with several

proteins within the GABAergic PSD, which in turn contribute in regulating the maintenance of proper function and localisation of $GABA_A$Rs [18] (Fig. 5).

Gephyrin has been first identified as a membrane associated protein and binding to polymerised tubulin at glycine postsynapse via its C-terminus domain [140]. Gephyrin trafficking has been investigated in detail and independent studies show that Dynein light chain (Dlc) 1 and 2 binds to gephyrin C-domain and contributes to transport of gephyrin along the microtubules. Consequently, Dlc is indispensable for normal localisation of gephyrin at GABAergic synapses [141]. Moreover, the membrane associated protein Profilin 1 and 2 contribute to anchor gephyrin at the actin cytoskeleton through its' binding at the E-domain. The complex formed between Profilin and gephyrin allows interaction with the microfilament adaptors of mammalian enabled/ vasodilator stimulated phosphoprotein (Mena/VASP) family [142, 143]. Mena/VASP is known to be regulating actin polymerisation; therefore regulating indirectly gephyrin transport (Fig. 5).

CB is a neuron-specific guanine exchange factor (GEF), belonging to the RhoGEF family and specifically activating the small Rho GTPase Cdc42. By binding to gephyrin E-domain, CB can translocate gephyrin from the cytoplasm to submembrane compartment [144]. Moreover, CB is important, if not essential, for gephyrin cluster formation at GABAergic PSD as in CB-deficient mice gephyrin clusters are undetectable in principal cells of the hippocampus. The impairment of gephyrin clustering in addition to a loss of $GABA_A$Rs expression lead to a decrease in GABAergic transmission and enhance LTP [145]. In hippocampal cell culture, over-expression of CB enhances gephyrin clustering at GABAergic PSD [146]. However elucidating the exact role and function of CB at the synapse remain complicated due to the existence of multiple splice variant of CBs. Disruption in mice and human CB gene *hPEM2* leads to multiple and variable clinical symptoms such as epilepsy, mental retardation and anxiety [145, 147, 148].

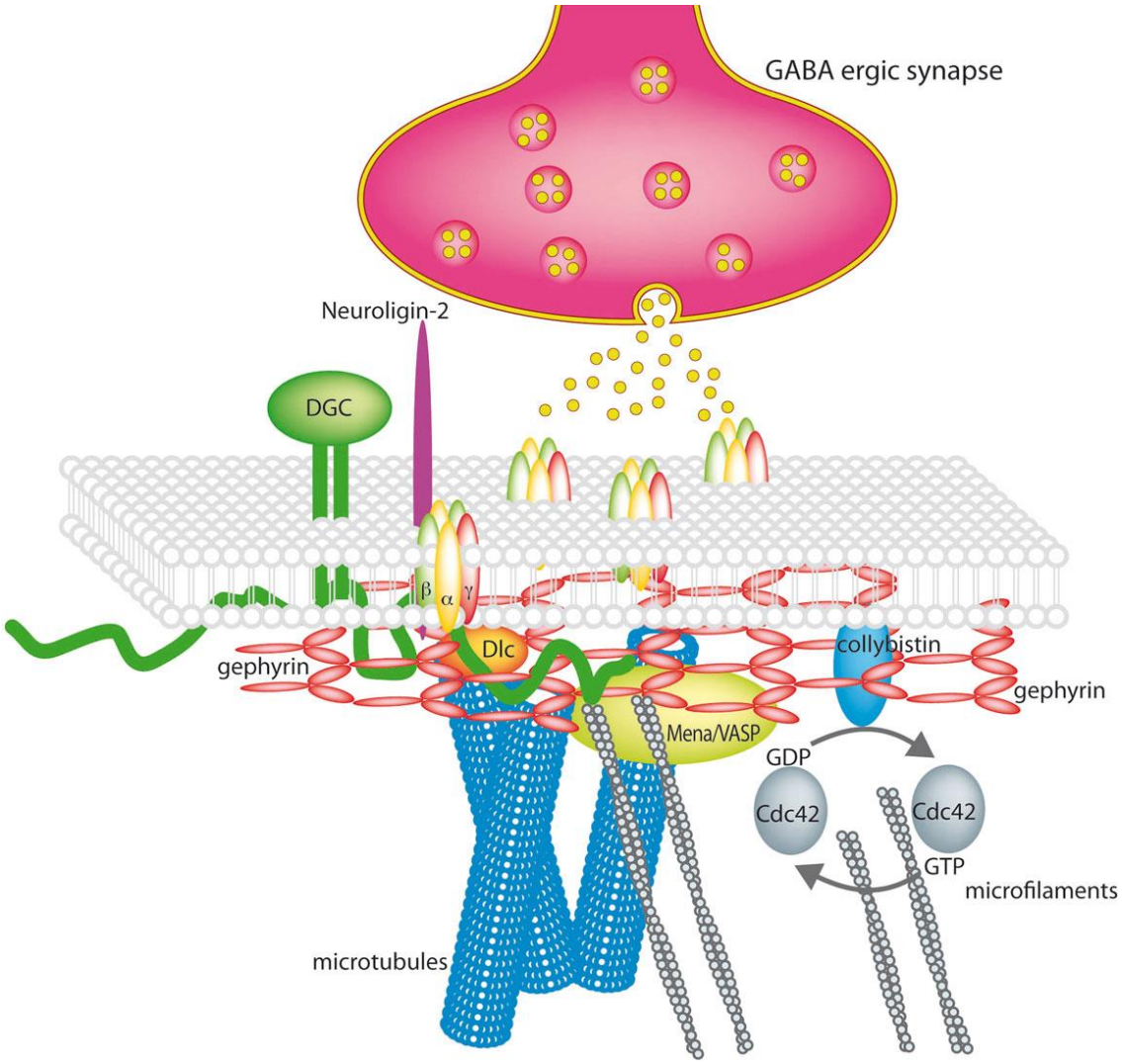

Figure 5: Schematic organization of GABAergic postsynaptic density (from Tretter and Moss 2008).

Dystrophin-glycoprotein (DGC) complex stabilizes the synapse. Neuroligin 2 contributes in this stabilization by contacting Neurexins at the presynaptic terminals. Synaptic $GABA_ARs$ are stabilised by their interaction with the scaffolding gephyrin at GABAergic post-synapses. Gephyrin is proposed to form a hexagonal lattice therefore a submembranous scaffold. Gephyrin, by its interaction with diverse proteins contributes to the structural organisation of the postsynaptic cytoskeleton. Collybistin interacts with gephyrin and activates Cdc42 which will initiate actin filaments remodelling. Cytoskeleton associated protein Dlc, profilin and Mena/VASP contributes in transportation of gephyrin along the microtubules [25].

NL2, through gephyrin and CB regulates the PSD assembly and maintenance at GABAergic synapses. NL2 is a trans-synaptic adhesion molecule anchored selectively at the GABAergic postsynaptic membrane and interacting with the presynaptic Neurexins [149]. This synaptogenic interaction is required and sufficient for initiating the formation and the functional maturation of new GABAergic synapses [25, 150]. NL2-KO mice show a reduction in GABAergic synapses in the hippocampus [19]. The interaction NL2 /gephyrin can be disrupted by the dephosphorylation of NL2 by Pin1, leading to a decrease in GABAergic synapse formation [94]. Interestingly, Pin1 can also bind directly gephyrin and enhance its ability to bind β subunits of glycine receptors [151]. Like for CB, the impairment of NL is associated to cognitive disorders such as schizophrenia and autism [21].

3.4 Gephyrin regulation by post-translational modifications

Gephyrin PTM is emerging as an important mechanism regulating gephyrin scaffolding properties: size and density, at GABAergic synapses (Table 1), thereby affecting structural and functional properties of GABAergic synapses [18]. It has been established long ago that gephyrin is a phosphor-protein [152]. Subsequently, it has shown that gephyrin undergoes proline-directed kinase phosphorylation at consensus serine residues. Subsequent to gephyrin phosphorylation Pin1 is recruited to induce a *cis/trans* conformational change, which is essential for gephyrin to bind β subunits of glycine receptors allowing the clustering of the receptors [151].

Subsequent studies identified another proline-directed kinase regulation of gephyrin via GSK3β signaling. GSK3β phosphorylates gephyrin at the serine 270 (S270) site to control the cluster density, by inducing proteolytic cleavage-induced turnover of gephyrin scaffold. The gephyrin^{S270A} mutation, mimicking the dephosphorylation status at the GSK3β site, increases gephyrin clustering density in primary hippocampal neurons [153]. Inhibition of GSK3β might occurs through activation of PI3K/Akt pathway followed by the reducing in gephyrin phosphorylation at S270 site. Therefore, reduced gephyrin phosphorylation favours the increase in $GABA_A$Rs containing α2 subunits and enhances GABAergic transmission. GSK3β-induced gephyrin phosphorylation is also relevant within the context of neuronal development, as

gephyrin phosphorylation by GSK3β site leads to reduced surface expression of $GABA_ARs$, causing neuronal hyper-excitability and dendritic shrinkage [154]. The S270 site seems to be modulated also by the Cycline-dependent kinase 5 (CDK5) in a CB-dependent manner. Inhibition of CDK5 reduces gephyrin cluster formation at GABAergic PSD [155].

Activated ERK causes gephyrin clustering downregulation at GABAergic synapses via phosphorylation at the Serine 268 site. Whereas, blocking ERK or the use of phosphorylation-defective gephyrin mutant constructs ($gephyrin^{S268A}$), in primary hippocampal neuronal cultures, enhances gephyrin cluster size and density [156].

Both GSK3β and ERK phosphorylations induce gephyrin cleavage and degradation via activation of Ca^{2+} dependent cysteine protease calpain 1 [156]. More recently, it has been demonstrated that gephyrin is a substrate for CaMKII and PKA phosphorylation at S305 and S303 respectively. These two kinases phosphorylate gephyrin in response to NMDA receptor activation to facilitate adaptive enhancement of GABAergic inhibition. The expression of $gephyrin^{S303A/S305A}$ blocks activity-dependent adaptations at GABAergic synapses [157].

In addition to kinases, gephyrin also interacts with phosphatases, such as protein phosphatase 1 (PP1). While the inhibition of PP1 induces a loss of synaptically localised gephyrin (reduction in size) with an enhancement of cytoplasmic gephyrin [158], the specific residues regulated by PP1 is still unclear. A new PTM has been identified in a unique study demonstrating gephyrin undergoing palmitoylation at two Cystein residues: C212/C284. This PTM seems to be part of the molecular mechanism necessary for anchoring and clustering gephyrin at GABAergic PSD. Palmitoylated gephyrin forms stable clusters at the PSD and potentiates GABAergic transmission; however, inhibition of this PTM reduces gephyrin clusters size [159].

Several acetylation sites have been identified on gephyrin using mass spectrometry analysis (MS/MS) of gephyrin from rat brain homogenate [18]. Several of the phosphorylation residues also a target for acetylation; however, the biochemical basis for such modifications is unclear. In addition, some of the lysine residues have also been shown to be acetylated on gephyrin [156]. A recent study showed that acetylation of gephyrin at K666 regulates SUMOylation of gephyrin (see next Section). Gephyrin is a substrate for SUMO-1 and SUMO-2, which act upstream of

phosphorylation and acetylation pathways to regulate gephyrin scaffolding at GABAergic synapses [Ghosh et al., Submitted].

PTM of gephyrin represent important cellular mechanisms for regulating the structure and function of GABAergic synapses in mature neurons; thereby ensuring homeostatic synaptic plasticity [18].

PTM sites	Effectors	Consequences on gephyrin	references
C212/C284	palmitoylation	Regulates gephyrin cluster number	[159]
S268	ERK1/2	ERK1/2 regulates gephyrin cluster size growth and density	[156]
S268	acetylation	unknown	[156]
S270	GSK3β and CdK5	GSK3β regulates gephyrin cluster number and CDK5 modulates gephyrin phosphorylation at S270 site	[153] [155]
S268E/S270E	Calpain-1	Phosphorylated gephyrin allow calpain cleavage	[156]
S303/S305	PKA/ CaMKII	Modulates gephyrin cluster number in activity-dependent mechanism	[157]
K666	acetylation	Important for gephyrin cluster formation	[Ghosh et al., Submitted]
S268E/K666A	Phosphorylation / deacetylation	Dominant negative gephyrin disrupting gephyrin clustering formation	
K148	SUMO-1	SUMOylation modulates gephyrin cluster size and its stability at the synapse	
K724	SUMO-2		

Table 1: Summary of gephyrin PTM

4. Post-translational modification by SUMOylation

4.1 Background

Small Ubiquitin-related Modifiers or SUMOs proteins are covalently attached to their substrates and the consequences of SUMOylation varies between substrates. SUMO PTM is a labile process, and often only a subset of proteins is SUMOylated at a given point making SUMO-modified proteins difficult to detect. Moreover, the roles of SUMOs are described to be amazingly versatile and uncover a variety of effects which are still being discovered. In brief, SUMO modulates protein-protein interactions, sub-cellular localisation, and activity and stability of its target substrate [160-164]. Therefore, it triggers multiple functional outcomes.

SUMOylation regulates diverse cellular processes, such as DNA repair [165, 166], transcriptional regulation [167-170], nuclear transport [171, 172] and cell migration [173-175].

SUMOylation can occur not only on nuclear proteins, but also on cytoplasmic proteins implicated in neuronal function. In neurons, SUMOylation regulates synapse development and plasticity. Therefore, SUMOylation affects many fundamental pathways often related to pathological conditions (see section 4.3) [164, 176, 177].

The SUMO proteins share ~18% sequence similarity with ubiquitin family members and each SUMO protein is ~100 amino-acids in length and 14KDa in mass. Four SUMO isoforms have been reported in literature. SUMO-1 shares about 50% homology sequence with SUMO-2 and SUMO-3 [178]. Whereas, SUMO-2 and SUMO-3 only differ by 3 amino-acids [179, 180]. SUMO-4 is similar to SUMO-2/3 but differs by the presence of a Proline instead of a Glutamine residue at position 90. Consequently, SUMO-4 is not processed [181] which make it the least characterized of the family members [182]. The expression of SUMO-1 and SUMO-2/3 is found in all human tissue whereas SUMO-4 seems to be restricted to the kidney, lymph nodes and spleen [163, 183].

Interestingly, SUMO proteins are indispensable for development and their role is crucial for embryonic development [184-186]. Alkuraya et al. demonstrate *sumo-1* as a key gene for mouse embryonic development and its knock-down is lethal [185]. Moreover, the data obtained by

Zhang et al. suggest the fact that *sumo-2* and *sumo-3* genes do not compensate for *sumo-1* gene loss but SUMO-2/3 proteins might compensate for SUMO-1 function [186]. Therefore, both studies highlight the importance of the role played by each SUMO paralogs. Although SUMO-1 and SUMO-2/3 isoforms recruit the same SUMO conjugation machinery, their often differ in their functionality [160, 187-189]. SUMO proteins are de-conjugated from protein substrates by sentrin proteases also called SENP. There seems to be a certain specificity of SENPs to specific SUMO proteins and specific pathological condition [190]. SUMO proteins can be further distinguished by their capability to form chains [188, 191]. SUMO-1 is unable to form chains, but SUMO-2/3 has the capability to form chains, via internal SUMOylation sites located at its N-terminus [188, 192-194]. While the SUMO-2/3 chain formation is also reversible, SUMO-1 can be often found at the end of a poly-SUMO-2/3 chain.

4.2 Overview of SUMOylation

The protein SUMOylation cycle (Fig. 6) is similar to the Ubiquitin cycle and is an energy consuming cascade made in three steps but their physiological consequences are quiet distinct.

SUMO isoforms are small polypeptides first translated as immature precursors. Free SUMO proteins are maturated by cleavage of their C-terminus via the SUMO-specific proteases (SENPs) in an endopeptidase reaction. Then get activated by the SUMO E1 activating enzyme in an ATP-dependent manner. The SUMO E1 enzyme is a heterodimer made of SAE1/SAE2 proteins that activate SUMO by forming a high energy thioester bond between its catalytic cystein and the C-terminal di-glycine (GG) of the SUMOs. Activated SUMO is transferred to the unique E2 conjugating enzyme, ubiquitin-like conjugating enzyme 9 (Ubc9). A trans-ester reaction allows Ubc-9 to conjugate SUMO to its substrate covalently through the di-glycine residue of the SUMO and the lysine residue (K) of the substrate. The E2 conjugation can be sufficient for SUMOylation to occur as long as the SUMO consensus sequence is present on the substrate [162]. However, the binding specificity to the K residue is often mediated by specific SUMO E3

ligase enzymes such as PIAS family [195] (see section 4.4). These ligases catalyse the transfer of the SUMO from Ubc9 to the target substrate protein.

SUMOylation takes place on a lysine residue, usually within the core of a consensus motif comporting ΨKXD/E where Ψ is a large hydrophobic amino acid such as Phenylalanine (F), tryptophan (W) or tyrosine (Y); K is the lysine residue, x is any amino-acid and D or E is any acid such as either an aspartate or a glutamine [196, 197]. However, not all consensus motifs are SUMO sites.

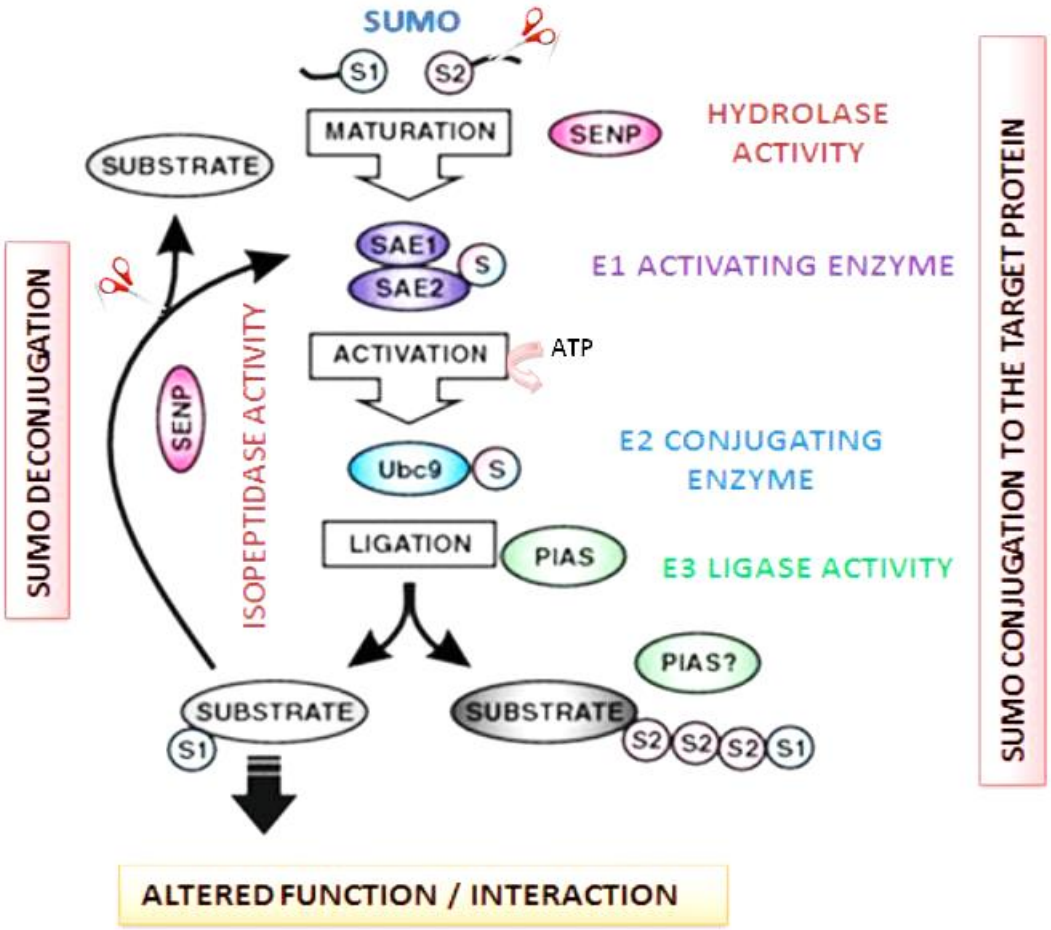

Figure 6: SUMO modification pathway (adapted from Rytinki et al. 2009).

SUMO proteins bind covalently to their substrates via a sequential action of three enzymes: E1 activating enzyme, E2 conjugating enzyme and E3 ligase enzyme. SUMO-2, but not SUMO-1, has the capacity to form a poly-chain. SENPs regulate maturation of the SUMO precursors and release conjugated SUMO from their substrate protein [195].

Some SUMO substrates possess longer sequences containing the SUMO consensus motif with additional elements such as negatively charged amino-acid dependent SUMO motifs (NSDM) or phosphorylation-dependent SUMO motifs (PDSM). In such cases, a phosphorylation site is found adjacent to the SUMO consensus motif: ΨKX(D/E)XXSP. SUMOylation at PDSM is enhanced by phosphorylation at the serine (S) residue. It has also been observed that the SUMOylation within the PSDM can convert transcription factors from activators to transcriptional repressors [198].

Interestingly, the SUMO conjugation does not necessarily requires this consensus motif but could also occur non-covalently at what has been called SUMO interaction motif (SIM). SIM motif is a hydrophobic core flanked by acidic amino-acid residues binding to a hydrophobic pocket at the surface of SUMO. Therefore, SUMO can modulate the activity and/or localisation of its target substrate. Interestingly, the SIM is also found on the SUMO E1 enzyme and PIAS family of SUMO E3 ligases. However, the E1 SIM functional relevance remains unclear; whereas in the PIAS family it is clear that SIM is not necessary for the E3 ligase activity but can be the binding site of SUMO.

The consequences of SUMOylation are dependent on the target protein. Therefore, three major effects retained are: first SUMO can increase the number of binding sites on its target. Second, it could mask a binding site and third it could induce change in conformation of its target protein [198].

4.2.1 Ubc9 and its neuronal function

In the mammalian system, Ubc9 is the unique SUMO E2 enzyme identified so far with the ability to recognise and conjugate SUMO to their substrate proteins [199, 200]. The lysine residue within consensus motif ΨKXD/E binds directly to Ubc9. One of the key regulators of Ubc9 substrate specificity is acetylation. Acetylation of Ubc9, on the lysine K65, helps Ubc9 to specially bind SUMO to substrates containing the minimal motif ΨKXD/E. This process can be reversed by the deacetylase SIRT1 which lead Ubc9 to conjugate SUMO with extended NDSM-containing substrates [201].

Ubc9 is the sole responsibility of SUMOylation conjugation and its expression is important for SUMOylation to happen. Interestingly, its expression level has been characterized via different molecular techniques as weak and spatio-temporally regulated. However, Ubc9 remains indispensable for embryonic development as its deletion is lethal [202].

4.2.2 SUMO proteases: SENPs

SUMOylation is a dynamic process controlled by the SUMO sentrines proteases (or SENPs) family, allowing only a small portion of proteins to be SUMO-modified at any given time [203]. The SENPs control the conjugation and de-conjugation of the SUMOs assuring the reversibility of the SUMOylation process [204] (Fig. 6).

The SENP family counts 6 isoforms: SENP (1-3 and 5-7) which possess a non-conserved N-terminal sequence thought to determine the substrate specificity and their subcellular localisation. SENP1, SENP6 and SENP7 are found in the nucleoplasm, SENP2 is confined to the nuclear pore complex and SENP3 and SENP5 are in the nucleolus [205]. They are responsible for maturating the free SUMOs precursors using their endopeptidase activity. In addition, the SENPs also catalyze the de-conjugation of the SUMO proteins from their substrates using their isopeptidase function. Furthermore, in the SUMOylation pathway all SENPs isoforms, with the exception of SENP1 and SENP2 show preference for SUMO-2/3 over SUMO-1. Moreover, SENP6 and SENP7 exhibit a high preference for SUMO-2/3 polychains [206].

SENPs in this way control the balance between SUMOylated and unSUMOylated proteins substrate. Therefore abnormal activities of the SENPs are related to pathologies, such as heart diseases and diverse types of cancers. Several compound targeting different SENP isoforms have been synthesized but their efficiency has not been clearly proven in clinical trials [207].

Among all the SENP isoforms, SENP1 is major enzyme implicated in several diseases. Under hypoxic conditions, SENP1 plays a crucial role in releasing the hypoxic-inducible factor 1α (HIF1α) from SUMO proteins. Thereby, SENP1 regulates the stability of HIF1α expression during hypoxia and avoid its ubiquitination and degradation [208].

4.3 PIAS family of SUMO E3 ligases

In general, PIAS proteins play an important role in the regulation of different transcription factors or synaptogenic molecules essential for keeping a balance of their activities in physiological and pathological conditions.

The specificity and enhancement of the SUMO-conjugation can be achieved through the recruitment of an E3 ligase enzyme such as PIAS family containing an SP-Ring domain. This domain is responsible for the SUMO E3 ligase function [195, 209]. The E3 enzyme binds to the E2~thioester SUMO complex to enhance SUMO conjugation *in vitro* and *in vivo* (Fig. 7).

The Protein inhibitor of activated STAT (PIAS) family consists of 4 different protein members: PIAS-1, PIAS-x (or PIAS-2), PIAS-3 and PIAS-γ (or PIAS-4). They share 5 conserved domains: Scaffold Attachment factor-A/B (SAP) domain, PINIT domain, Zn finger /SP-Ring domain, SUMO interactive motif (SIM) and C-terminus or Serine/Threonine (S/T) C-terminal rich region [195] (Fig. 7).

The PIAS family proteins are described as regulators of gene-activation pathways in non-pathological [195, 210] and pathological conditions involving an immune response [211, 212]. They were initially discovered as negative regulator of cytokines signaling that inhibit the Signal transducer of activated transcription (or STATs) transcription factor and recently as SUMO E3 ligase enzymes [195, 209, 210]. The function of transcriptional regulator is closely interlinked with the SUMO ligase function [210].

The N-terminus SAP domain of PIAS is known to be involved in sequence and structure specific DNA binding (Fig. 7F), in addition to recognizing protein interactors, the SAP domain has been shown to be necessary for binding and repressing STAT-1 activity by PIAS-γ [213]. Downstream of SAP is the PINIT domain, which is required for subcellular localization of PIAS-3 [214] and for PIAS-3 inhibition of the transcription factors STAT-3 [215, 216] and Microphthalmia transcription factor (MITF) [217]. Moreover, the PINIT domain has been shown in some cases to be important for substrate binding. The PINIT domain changes the conformation of the PIAS protein allowing the attraction of the E2~thioester SUMO complex and activation of conjugation

[198, 218]. The PINIT domain of PIAS-γ stimulates SUMO modification by SUMO-2 in some cases [195].

The RING-type zinc-binding structure or SP-Ring domain is essential for SUMO E3 ligase function and bind directly to Ubc9 [195, 209]. SP-Ring activity has variable functional consequences according to the protein substrate. It has been shown to alter the substrate function, to regulate its subcellular localisation and to modulate the interaction of the substrate with other proteins partner (Fig. 7A-D).

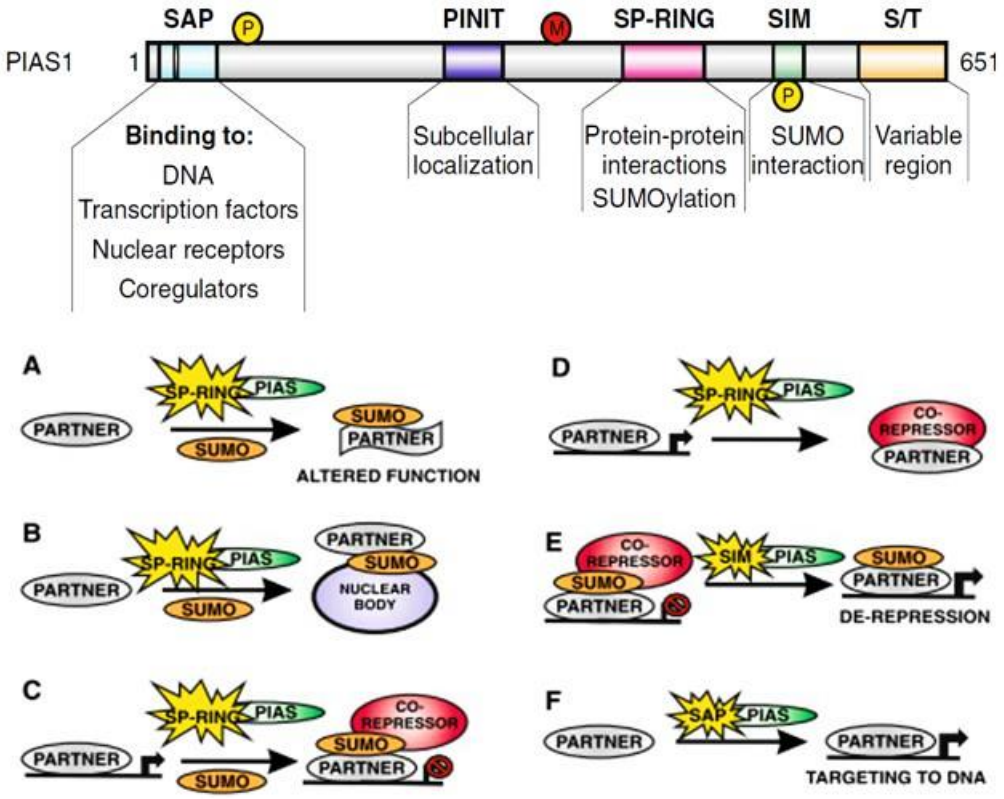

Figure 7: PIAS proteins and their different domains' functions. (Adapted from Rytinki et al. 2009). Schematic structure of PIAS-1 protein with the 5 different conserved domains. Schematic models of SP-Ring, SIM and SAP-dependent mechanisms in transcription regulation.

PIAS proteins harbour a SIM motif between the SP-Ring motif and the C-terminus. The SIM motif provides to the protein the capacity to bind non-covalently the free SUMOs proteins without being necessary for the SUMO E3 ligase function [198]. Moreover, by binding to the substrate it can release the last one from its protein partner and allow transcription to happen, in the case of a transcription factor (Fig. 7E).

Compared to the other domains, the S/T C-terminal region is the least conserved domain absent in PIAS-γ. It is a variable region which can serve as protein interaction domain. Interestingly, PIAS family of proteins are themselves subject to PTM. PIAS-γ is regulated by SUMOylation to enhance its enzymatic function. Ubiquitin E3 enzymes mediate proteasomal degradation of PIAS. During oxidative and nitrosative stress, PIAS-3 is target for S-nitrosylation enhancing its affinity for the Ubiquitin E3 enzyme tripartite motif-containing 32 (TRIM32) followed by ubiquitination and degradation of PIAS-3 [219].

4.4 SUMO modification in neurons

The importance of SUMOylation in neurons is still emerging; however, this PTM seems to have diverse implications, from transcriptional regulation in the nucleus to axonal RNA trafficking and synapse plasticity (Fig. 8) [190, 220, 221].

It has been pointed out that the level of Ubc9 mRNA expression and SUMO-1 conjugation are the two mains SUMOylation members playing a critical role in neuronal development [222].

Moreover, in neurodevelopment PIAS-3 is indispensable for photoreceptors differentiations in mouse retina. PIAS-3 promotes the formation of rods photoreceptors through SUMOylation of specific transcription factors. PIAS-3 SUMOylation of the transcription factor NR2E3 lead to activation of gene transcription involved in the favour of rod photoreceptor differentiation. In parallel, gene transcription necessary for cone-differentiation are repressed. This indicates the complexity via the fact that SUMOylation can regulate substrate specificity and differentiation of neuronal subtypes [220, 223].

SUMOylation is an important regulator of synaptogenic proteins such as CASK and transcription factor myocyte enhancer factor 2A (MEF2A) thereby promoting or repressing synaptogenesis. CASK is a Ca^{2+}/calmodulin-dependent serine protein kinase essential for formation, stabilisation and maintenance of dendritic spines. When CASK is SUMOylated by SUMO-1, at K679 site, it impairs spine formation in hippocampal neurons [224]. However, SUMOylation of MEF2A, by SUMO-1 (at K403 residue) and PIAS-x, promotes postsynaptic dendritic claw differentiation by repressing its transcriptional activity [225]. This last study suggests that PIAS-x might have a crucial role in the establishment of neuronal connectivity. Moreover, these results demonstrate that, depending on SUMO-1 protein substrate, SUMOylation can induce opposite effects on synaptogenesis (Fig.8).

SUMO not only plays a role in synaptogenesis but has an important role in the regulation of synaptic plasticity by modulating presynaptic and postsynaptic mechanisms. Depending on the stimulus applied (Kainate or AMPA), it has been shown that SUMO might either up or downregulate presynaptic glutamate release. Nevertheless, these studies allowed identification of the SUMO substrates involved in controlling neurotransmitter release. Interestingly, at presynaptic sites group III mGluRs carry a SUMO consensus motif on their C-terminus and can be SUMOylated *in vitro*. However, SUMOylated mGluRs have not been detected directly in neurons. More work would be needed for understanding the relevance of SUMOylation of the mGluRs and to address whether they effectively influence synaptic release [221].

At postsynaptic sites, SUMO regulates neurotransmitter receptors function and trafficking at glutamatergic PSDs in response to various forms of plasticity [221, 226]. Induction of long-term potential or depression (LTP/LTD) modulates the levels of SUMO-conjugated substrates, such as kainate and AMPA receptors (AMPARs).

The kainate receptor GluK2 (formerly GluR6) is a SUMO-1 substrate, and binds both Ubc9 and PIAS-3. Upon kainate stimulation, GluK2 is SUMOylated at the lysine residue K886 on its C-terminus contributing to its endocytosis at mossy fibers-CA3 synapses. Interestingly, SUMOylation of GluK2 is promoted by phosphorylation of the subunit by PKC at S868. Furthermore, phosphorylation and SUMOylation collaborate to regulate kainate receptor trafficking as endocytosis of GluK2 is an essential mechanism for LTD [221, 226].

In parallel, elevation of SUMOylation might be implicated in AMPARs upregulation as their insertion and removal is regulated by SUMOs. However, to our knowledge, no reports confirm the direct SUMOylation of AMPARs. Additionally, several proteins downstream of glutamate receptors are subjected to SUMOylation in turn regulating AMPARs function. During synaptic scaling or induction of LTP, SUMOylation of the immediate early gene product Arc regulates activity-dependent trafficking of AMPARs [221].

Beside modulation of the pre- and postsynaptic response, SUMO regulates the general excitability of the neurons. Neuronal excitability is in part governed by voltage-gated ion channels such as potassium channels such as Kv2.1, K2p1 and K1.5.

Taking together, these observations underscore the variety of effects due to SUMOylation of neuronal proteins. Even SUMO modification has been extensively investigated in cancer [227-229], essentially for its contribution to the regulation of cell proliferation; SUMOs have been recently connected to a growing number of neurodegenerative diseases [190, 230-235].

For example, impairment of episodic and fear memory is observed after silencing by knock-down (KD) *Sumo-1-3* in mice hippocampal and cortical neurons. Moreover, this impairment can also be observed following the KD of the E3 ligase PIAS-1 and decrease in CREB SUMOylation in hippocampal CA1 neurons [236, 237]. Interestingly, CREB SUMOylation plays an important role in sustained long-term memory by enhancing its DNA binding function, leading to an increase in *bdnf* transcription [236]. Hence, the SUMO pathway not only has substrates at dendritic synapses, but also links synaptic adaptations to specific transcriptional program within the nucleus.

Interestingly, the global level of SUMOylation fluctuates according to an external stimulus such as cellular stress [231, 238-240]. SUMO-2/3 seems to be the more sensitive to various stress (osmotic stress, heat shock and oxidative stress) as the level of overall SUMO-2/3 conjugation is strongly raised compared to SUMO-1 conjugation level [187]. In the case of ischemic injury both SUMO-1 and SUMO-2/3 are increased and this effect is believed to be beneficial for recovery

[241, 242]. Over-expressing SUMO proteins or the use of Ubc9 transgenic mice, rising the general SUMO level, contribute to the protection against brain ischemia [240].

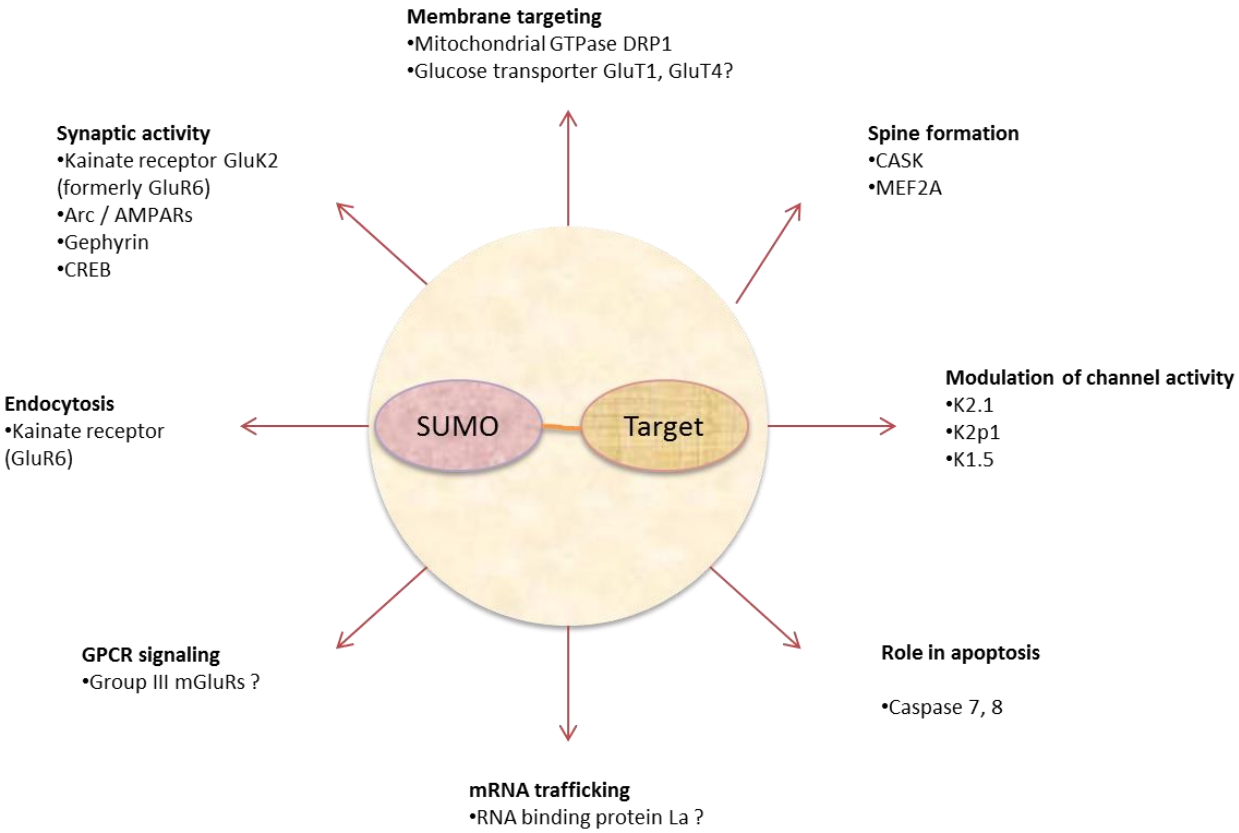

Figure 8: Emerging roles of proteins SUMOylation in neuronal function. (Adapted from Martin et al 2007)

SUMOylation regulates various aspects of neuronal function and morphology. A question mark highlights the speculative and unconfirmed proteins functions. DRP1, dynamin-related protein 1; GluR6a, glutamate receptor 6a; GLUT, glucose transporter; GPCR, G-protein-coupled receptor.

5. Cross talk between different cellular pathways

There is functional cross talk between the main PTM, including phosphorylation, SUMOylation, ubiquitination and acetylation for functional regulation [203, 243]. It has been known for some time that SUMOylation prevents its substrate from degradation, which is contrary to ubiquitination. However, SUMOylation and ubiquitination can influence each other to facilitate degradation of subset of proteins [244, 245]. A SUMO-targeted ubiquitin E3 ligase (STUbls), such as RNF4, can recognize, via its SIM motif, poly-SUMOylated substrates thereafter initiating their ubiquitination and proteasomal degradation [195].

E3 ligase PIAS proteins are also subjected and regulated by ubiquitination. PIAS-1 and PIAS-γ have been both reported to be ubiquitinated by the E3 ubiquitin ligase hSiah2 and Trim32, respectively. Therefore, direct them to protein degradation. However, ubiquitination onPIAS-2 and PIAS-3 alters their subcellular localisation [195].

SUMOylation is subjected to the PTM via acetylation and ubiquitination as the three SUMO paralogs compete for the same lysine residue on a substrate (Fig. 9). Phosphorylation does not compete for the same site than SUMOylation but can act as a positive or negative regulator of SUMOylation [203]. In an inverse manner, SUMOylation can also modulate the phosphorylation state of a substrate [243, 246]. As described above in 4.2 section, SUMO substrate protein carrying a PDSM can be phosphorylated at a site close to the SUMO consensus site leading to an enhancement of SUMOylation of those proteins [198]. However, phosphorylation can also lead to inhibition of SUMOylation to influence transcriptional regulation.

The transcription factor MEF2A is phosphorylated within the PDSM motif thereby enhancing its SUMOylation, and inhibiting its transcription activity[225]. In contrast, dephosphorylation of MEF2A by calcineurin inhibits it SUMOylation. Moreover, in addition to phosphorylation and SUMOylation, MEF2A can undergo acetylation on the same Lysine residue targeted by SUMO. Interestingly, the switch from SUMOylation to acetylation is made by calcineurin. Moreover, acetylation process is promoted by phosphorylated histone deacetylases, hence regulating its transcriptional activity [198, 203]. Both deSUMOylation and acetylation lead to inhibition of synapse formation.

Phosphorylation of transcriptional regulators such as JUN, FOS or p53 prevents their SUMOylation thereby increasing their transcription activity [203]. Thus, both phosphorylation and SUMOylation regulate gene transcription. Moreover, this control of SUMOylation can be mediated by the MAPK pathway for certain subset of substrates [197].

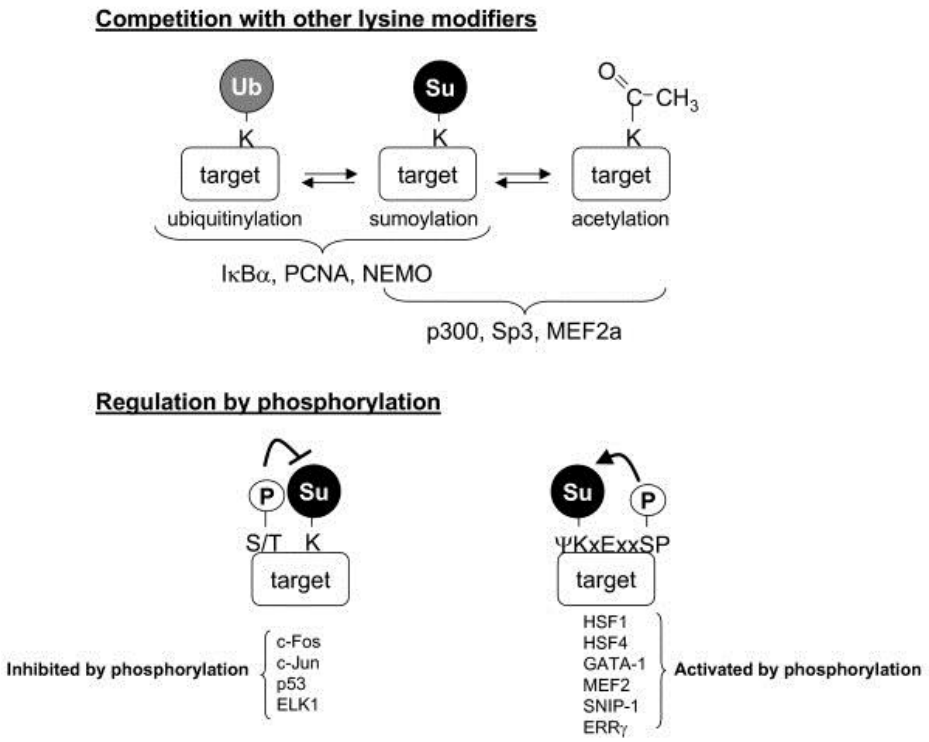

Figure 9: Regulation of SUMOylation trough target modification. (Bossis and Melchior, 2006)

Competition of acetylation and ubiquitination at the same lysine residue induces inhibition of SUMOylation. Interestingly, Ubiquitination and SUMOylation can join their force for regulating specific substrate proteins. Phosphorylation acts on a different site than SUMOylation and prevents or promotes SUMOylation of the targeted substrate [203].

6. GABAergic transmission in brain pathology

Understanding the regulation of synaptic connectivity open new perspectives for elucidating patho-physiological mechanisms underlying psychiatric disorders and neurological diseases.

Two non-related diseases such as schizophrenia and stroke are accompanied with a reduction in tonic inhibition, consequence of a reduction in extrasynaptic $GABA_A$Rs; in addition to a reduction in synaptic receptors. Moreover, the alteration in GABAergic tonic inhibition and a decrease in mIPSCs induce hyper-excitability of the principal neurons [6]. Therefore, modulations of $GABA_A$Rs trafficking play an important role in physiological and pathological conditions.

Phosphorylation is the major PTM happening at GABAergic synapses and plays a crucial role in trapping the receptors at the synaptic sites. Reciprocally, the receptor dephosphorylation through activation of different kinases regulates receptors endocytosis. Endocytosis regulation depends on the phosphor-state of the two relevant $GABA_A$Rs subunits: β and γ2 subunits. Those subunits are subjected to different kinases, phosphatases and their respective adaptors. Clinically, a reduction in the kinase PKC-mediated phosphorylation of $GABA_A$Rs β subunits induces a dramatic loss of GABAergic inhibition contributing in the prolongation of seizures. Interestingly, dephosphorylation of $GABA_A$Rs γ2 subunits by generating knock-in mice with point mutation in those two tyrosine residues Y365/Y367 leads to embryonic lethality. Therefore, underscore the possibility that excessive GABAergic excitation is detrimental during early development [15].

However, a reduction in $GABA_A$Rs expression and function is also related to numerous diseases such as Huntington and non-neuronal development. In Huntington disease $GABA_A$Rs number inserted at the plasma membrane and GABAergic transmission are reduced contributing to neurodegeneration.

In pathology such as ischemic stroke, inhibitory synapses are downregulated via reduction of GABAergic transmission at the pre- and post- synaptic levels [247, 248]. More precisely, at inhibitory synapses, cerebral ischemia or OGD (*in vitro* model of ischemia) decreases cell surface

expression of $GABA_AR$ [247] by reducing the mRNA and proteins levels of different $GABA_AR$ subunits such as α1, β2, β3 and γ2 [249]. This downregulation of the mRNA level of the different subunits is via a calpain-dependent mechanism [248] and can be prevented by blocking NMDA and non-NMDAR [250]. Moreover, OGD decreases surface expression of $GABA_ARs$ by enhancing the de-phosphorylation of $GABA_AR$ β3 subunits therefore inducing internalisation of the receptors involved in the induction of neuronal cell death [73]. Moreover, extrasynaptic $GABA_ARs$ expressions are decreased, in the pre-infarct zone, followed by a reduction in tonic inhibition thereby favour functional recovery [251]. These observations highlight the importance of $GABA_ARs$ trafficking at the plasma membrane under physiological and pathological conditions.

However, regulation of $GABA_ARs$ surface expression is not only regulated by phosphorylation but also through the interaction with the main scaffolding protein gephyrin. After an ischemic insult it has been highlighted the loss of binding between $GABA_ARs$ and gephyrin might contribute towards the loss of synaptic receptors. Gephyrin expression is decreased following OGD and its oligomerization might be affected. In addition, gephyrin is actively removed from GABAergic synapses by the action of protease calpain1 to limit GABAergic inhibition [252]. Interestingly, gephyrin cleavage is facilitated by phosphorylation of gephyrin by ERK and GSK3β [156]. Given that phosphorylation and SUMOylation pathways converge on gephyrin to regulate its scaffolding properties [Ghosh et al., Submitted], it is therefore possible that both SUMOylation contributes to gephyrin scaffolding and in turn $GABA_ARs$ function after OGD.

Furthermore, SUMO modification is dynamic and SUMOylation levels are correlated with protection against cellular stress [190, 239]. In response to protein-damaging stimuli, such as ischemic stroke, the levels of SUMO-1 and SUMO-2/3 conjugation of cellular protein increases [253]. This suggests that SUMOylation might be neuroprotective against ischemic stroke as it can decrease neuronal excitability via the regulation of surface expression of the kainate receptor GluK2. Kainate receptors are responsible for glutamatergic excitotoxicity and by promoting its endocytosis SUMOylation might decrease the excitotoxic effect.

Importantly, these studies highlight the importance of $GABA_ARs$-mediated transmission in normal and pathological brain function by controlling neuronal excitability.

II/ Hypothesis and aims of the thesis

Research in the past decade has identified the scaffolding molecule gephyrin at the heart of mechanism underlying GABAergic synaptic plasticity. The regulation of gephyrin scaffolding is a direct correlate for $GABA_ARs$ function at synaptic sites. Gephyrin is regulated by diverse molecular mechanisms such as RNA splicing, interacting proteins partners and PTM [18]. Gephyrin interaction with $GABA_AR$ subunits has been described in literature [254] ; however, the regulatory basis for this interaction is still unclear.

Identification and characterization of cellular pathways that converge onto the gephyrin scaffold has shed some light into the molecular basis underlying adaptations at GABAergic synapses. Gephyrin phosphorylation by GSK3β at S270 and ERK1/2 at S268 influences GABAergic synapse density and size respectively. Furthermore, a recent study identified SUMOylation of gephyrin as a new PTM event that influences its phosphorylation status, whereby affecting its scaffolding property. The upstream signals that activate GSK3β, ERK1/2 to phosphorylate gephyrin are currently unclear. Similarly, there is very little understanding about the upstream events regulating the protein SUMOylation machinery. The main tenet of this thesis proposal is to demonstrate that BDNF signaling influences gephyrin PTM(s) to facilitate structural and functional adaptations at GABAergic synapses by interacting with gephyrin SUMOylation.

We specifically hypothesize that TrkB signaling downstream of BDNF activates ERK and GSK3β along with the SUMO pathway to modify the gephyrin scaffolding properties. To test our hypothesis we employed a multidisciplinary approach combining cellular biology, biochemistry, molecular pharmacology and electrophysiology in both primary hippocampal neurons and organotypic hippocampal slice cultures.

Study I: **BDNF regulates PIAS-3 function for modulating gephyrin scaffolding at GABAergic postsynaptic sites.**

Gephyrin undergoes SUMOylation and its aggregation properties can be modulated by the SUMO E3 ligase enzyme called PIAS-3. E3 enzymes have the general properties to enhance specificity of the SUMO-conjugation. We have identified and characterized SUMO-1 and SUMO-2 conjugation sites on gephyrin. Mutation of the SUMO-1 and SUMO-2 lysine residues on gephyrin enhances its scaffolding property. Hence, we reasoned that gephyrin scaffolding via SUMOylation must influence plasticity change at GABAergic synapse. To address this issue we dissected the mechanistic basis for BDNF induced gephyrin clustering change at GABAergic synapse.

Study II: **SUMOylation-defective gephyrin mutant stabilizes GABAergic synapses in post-ischemic CA1 hippocampal neurons**

Ischemia is a condition well known to activate the protein SUMOylation machinery and to strongly upregulate BDNF transcription. The aim of this study was to demonstrate that BDNF influences the SUMO pathway to affect gephyrin scaffolding at GABAergic synapses in pathology such as ischemia. The major tenet of our hypothesis is that in ischemic conditions BDNF signals through TrkB receptors to influence GABAergic synapses via gephyrin regulation. In order to test this idea, we employed organotypic hippocampal slice cultures to induce ischemia by transient oxygen-glucose deprivation (OGD). Using a combination of morphology and electrophysiology combined with pharmacology manipulation we uncover the molecular events that impinge on GABAergic postsynaptic apparatus in response to ischemic insult.

III/ Results

Chapter 1: BDNF regulates PIAS-3 function for modulating gephyrin scaffolding at GABAergic postsynaptic sites.

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ZT performed the experiments, data analysis, and participated in writing the manuscript. S.F. performed some of the biochemical experiments.

Abstract

The postsynaptic scaffolding protein gephyrin is a crucial component at GABAergic postsynaptic sites ensuring the efficiency of neurotransmission. Multiple independent studies demonstrated the implication of gephyrin PTM in remodelling gephyrin scaffolding properties thereby contributing to GABAergic synaptic plasticity. Here, we focused on SUMOylation of gephyrin and the regulation of gephyrin clustering by BDNF in primary hippocampal neuronal cultures. We identified BDNF as the upstream signaling molecule regulating the subcellular localisation and the function of specific members of the SUMO pathway, especially the E3 ligase PIAS-3. Moreover, using gephyrin mutant constructs that cannot be SUMOylated, we show BDNF inefficiency to downregulate gephyrin cluster. Additionally, we show that PIAS-3 affects gephyrin postsynaptic clustering via both a SUMO-dependent and a SUMO–independent function. Moreover, we provide evidence that gephyrin SUMOylation is dependent on its phosphorylation status by ERK1/2 and GSK3β. Importantly, both PIAS-3-dependent mechanisms are under the control of BDNF signaling. Altogether, these results demonstrate the importance of gephyrin phosphorylation and SUMOylation for regulation of GABAergic function by BDNF.

Introduction

The neurotransmitter GABA mediates most inhibitory neurotransmission in the CNS by acting on its receptors $GABA_A$ ($GABA_ARs$) and $GABA_B$ receptors. The $GABA_ARs$ are pentameric ligand-gated chloride channels that are assembled from a repertoire of 19 different subunits [23]. Deficits in GABAergic transmission have been implicated in several neuro-developmental and neuropsychiatric disorders [8, 255-257]. Hence, $GABA_ARs$ represent a common class of pharmacological drug targets for the treatment of insomnia and anxiety disorders.

In the past two decades it has become increasingly clear that $GABA_ARs$ are regulated by a multitude of cellular signaling processes, such as transcription, post-translational RNA editing and PTMs. In addition, several $GABA_ARs$ interacting proteins, especially its main scaffolding protein gephyrin, have been identified as substrates for diverse PTMs [8, 20, 157]. However, the molecular underpinnings of synaptic plasticity at GABAergic synapse are poorly understood at present.

In recent years it has become evident that multi-functional protein gephyrin is both a main organizer of GABAergic postsynaptic sites and a facilitator of downstream signaling events [20, 113, 124, 127, 131, 258]. Gephyrin undergoes PTMs by diverse signals transduction pathways to regulate its scaffolding properties [18, 131]. Our own research has demonstrated that gephyrin phosphorylation by GSK3β and ERK1/2 signaling pathways alter the scaffold density and size respectively, in turn influencing GABAergic mIPSCs amplitude and frequency [93, 153]. Subsequently, it was demonstrated that gephyrin is a novel substrate for SUMO-1 and SUMO-2. Identification of specific SUMOylation residues on gephyrin demonstrated the significance of this modification for its scaffold formation at GABAergic synapse and for modulation of GABAergic strength [Ghosh et al., submitted]. In the same study it was also shown that complex cross-talk between phosphorylation, acetylation and SUMOylation determine gephyrin scaffolding dynamics. Expression of a gephyrin dominant negative mutation that disrupted gephyrin clustering in neurons showed significantly reduced mIPSCs amplitude and frequency [Ghosh et al., submitted], suggesting that gephyrin scaffold is essential for majority of the GABAergic synapses to function.

Signals upstream of cellular kinases and SUMO pathways and their relevance for GABAergic inhibition under pathophysiological conditions remain unclear. One of the potential upstream signaling molecules known to modulate GABAergic inhibition is the brain derived neurotrophic factor (BDNF). As the most abundant neurotrophin, BDNF has been shown to play an important role in synaptic plasticity and circuit maturation during brain development; however, it is still unclear how BDNF influences synaptic plasticity change in adult brain [99, 106, 259, 260]. At GABAergic synapse BDNF has been shown to exhibit a bidirectional effect depending on the duration of its application [99, 261]. Short-term application (acute) of BDNF reduces GABAergic postsynaptic responses [101, 102, 108, 113] with a significant reduction in the cell surface expression of α2 $GABA_ARs$ subunit [101] and downregulation of gephyrin clusters [102, 108]. The acute effect of BDNF is via the high affinity receptor, tropomyosin-related kinase B (TrKB) [102, 108], and leading to internalization of $GABA_ARs$ at the synapse. In contrast, chronic BDNF treatment was shown to strengthen GABAergic transmission accompanied by an increase in the cell surface expression of $GABA_ARs$ [115, 116, 262, 263]. The molecular mechanism underlying BDNF induced short- and long- term synaptic plasticity changes remain to be identified. Independent studies have suggested the involvement of postsynaptic mechanisms; nevertheless, it is possible that this regulation at the level of $GABA_ARs$ is via the regulation of gephyrin scaffolding.

In support of this hypothesis, it was shown that acute BDNF application regulates the availability of postsynaptic $GABA_ARs$ [101, 107, 108] by decreasing gephyrin interaction, leading to the internalization and degradation of the receptors [102]. Given the importance of gephyrin PTM via phosphorylation and SUMOylation for its scaffolding dynamics, in the current study we examined the role of BDNF signaling upstream of these signaling cascades.

To test this idea we used primary hippocampal neurons and employed molecular biology, biochemistry and pharmacology to demonstrate that BDNF acts upstream of the SUMO pathway. BDNF regulates the sub-cellular localization and function of specific members of the SUMO pathway to influence gephyrin scaffolding. In particular, BDNF impinges on the function of E3 ligase PIAS-3, which in turn influences gephyrin scaffolding via two independent pathways. The two independent pathways require the activation and inactivation of TrkB receptors specifically. Finally, we demonstrate that SUMOylation and phosphorylation of gephyrin at specific residues

converge at the level of PIAS-3 function. Together, our data identifies a novel mechanism involving BDNF-regulation of SUMOylation pathway for influencing GABAergic post-synapse plasticity.

Materials and Methods

Plasmids

EF1a-eGFPC2-gephyrin has been described earlier [93, 156], pCI-FLAG-(SENP-1, SENP-2, SENP-3, SENP-5, SENP-6) and pCS3±6xmyc (PIAS1, PIAS2α, PIAS2β, PIAS4) have been described earlier [264]. pCS3±6xmyc-PIAS-3 (1-584AA and C299S/H301A SP-Ring mutant) have been described earlier [265]; eGFP-PIAS-3 was a kind gift from Prof. Johar Yogil (Hebrew University, Jerusalem). The plasmids pCS3±6xmyc–PIAS-3 (1-273AA, 274-392AA, 274-584AA, 393-584AA and 416-584AA) were kind a gift from Prof. Fang (Rutgers University, New Jersey, USA). pCS3±6xmyc-PIAS-3(1-406) was generated by deleting the C-terminus domain from pCS3±6xmyc-PIAS-3. pCS3±6xmyc-PIAS-3 (I200V/T201A or PINIT mutant) and pCS3±6xmyc-PIAS-3 (I200V/T201A, C299S/H301A or PINIT/Ring mutant) were generated using site directed mutagenesis in pCS3±6xmyc-PIAS-3 and sequence confirmed. eGFP-gephyrin (S268E and S270E) are described earlier [93, 156]; eGFP-gephyrin SUMO-1 and SUMO-2 site mutations (K148R and K724R) are described in [Ghosh et al., submitted].

Primary hippocampal neuronal culture

All animal experiments were approved by the cantonal veterinary office of Zurich. Dissociated embryonic (E17-E18) Wistar-rat hippocampal primary mixed cultures were prepared as described earlier [93, 156]. They were maintained in the culture media containing MEM (Gibco), 15% Nu-serum (Becton-Dickinson,355500), B27 supplement (Invitrogen), 1M HEPES (pH7.2; 15mM), glucose monohydrate (0.45%), 1mM Na-pyruvate and 2mM L-Glutamine. The cells were transfected following the protocol described in T. Buerli et al. 2007 [266], at 8 days *in-vitro* (DIV) with 1μg total plasmids DNA with up to a total of 3 different plasmids transfected simultaneously. We used Lipofectamine 2000 (Invitrogen, 11668-019), CombiMag (Oz Biosciences, CM21000) and OptiMEM medium (Invitrogen, 31985-070) as per the protocol.

Immunohistochemistry of primary cells culture

7 days post-transfection the cells (8+7DIV) were fixed in 4% PFA for 10min then permeabilized for 5min with 0.1% TritonX100 in 10% Normal goat serum (NGS, BioRad, C07SA) and PBS pH 7.4. The cells were quickly washed with PBS (pH7.4) before being labelled with the appropriate primary antibody cocktail (antibodies with 10%NGS and PBS) for 90min. After three washes of 10min each with PBS the secondary detection was achieved with the secondary antibody mixture supplemented with DAPI (1:1000) for 30min. The cover-slips were mounted with Dako Fluorescence Mounting medium (Dako North America, Inc.).

Antibodies

Mouse anti-Gephyrin (1:1000, clones mAb7a, Synaptic Systems #147021), rabbit anti-SUMO-1 (1:250, Epitomics#1563-1), mouse anti-SUMO-1 (1:100, SantaCruz#sc-5308), rabbit anti-SUMO-2/3 (1:250, Cell signaling #4974), rabbit anti-SUMO-2/3 (1:250, Epitomics #2970-1), mouse anti-PIAS-3 (1:500, Sigma #P0117), rabbit anti-vGAT (1:2000, Synaptic Systems #131011); mouse anti-Myc tag (1:5000,Roche #11667149001), rabbit anti-Myc tag (1:5000, Cell Signaling #2278S), mouse anti-FLAG tag (1:5000, Sigma Aldrich #F3165). All the secondary antibodies were from Jackson ImmunoResearch: Goat anti-Mouse Cy3 IgG (1:500, #115165), Goat anti-Mouse IgG Cy5 (1:500, #115175), Goat anti-Rabbit IgG Cy3 (1:500, #111165) and Goat anti-Rabbit IgG Cy5 (1:500, #111175).

Pharmacological treatments

Transfected cells were treated 90min with hBDNF (10ng/mL, Alomone Labs #B-250), NT-3 (10ng/mL, Alomone Labs #N-260), or NT-4 (10ng/mL, Alomone Labs #N-270), and/ or a BDNF scavenger: rh TrKB-Fc Chimera (1µg/mL, R&D Systems #688-TK-100). Otherwise the cells were treated overnight with ERK 1/2 inhibitor: PD98059 (25µM/mL, Calbiochem#513000) or GSK3β inhibitor: GSK3βIX (5µM/mL, Calbiochem #328007) or DMSO (equal volume; Sigma D2438).

Image analysis and quantification

All imaging were acquired on confocal laser scanning microscope (LSM 710, Carl Zeiss) with objective lens of 40x or 63x lens (NA 1.4) with a pinhole set at 1 Airy unit and a pixel size of 0.13µm. For each condition, images from a minimum of 9 cells from 3 independent batches of neuronal culture were acquired using a z-stack (3-5 steps at 0.5µm per step size). From each cell a dendritic segment was taken for analysis. Image analyses were performed with a custom written analysis for Image J software using maximal intensity z-projected images.

Gephyrin clustering size area and density were analysed 7 days post-transfection in hippocampal primary neuronal culture following the protocol previously described [93, 156]. Only gephyrin cluster apposed to the presynaptic marker vGAT were considered for quantitative assessment. The generated data are then plotted using Excel software and Graphpad Prism software.

Statistical analysis

Morphological changes of eGFP-gephyrin clustering size were assessed using a pair-wise Kolmogorov-Smirnov (KS) cumulative distribution analysis using the online platform http://www.physics.csbsju.edu/stats/KS-test.n.plot_form.html. When multiple groups were compared a One-Way ANOVA followed by a Tukey test (PRISM software) was performed. Whereas changes in eGFP-gephyrin clustering density were assessed, using PRISM software, by either a Student T-test or a One-Way ANOVA.

HEK 293 cell cultures and transfection

Human Embryonic Kidney (HEK293) cells were maintained at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Gibco 41966-029), supplemented with 10% fetal calf serum (FCS, Gibco #10270-106). They were transfected, 24h post-plating, with either 1µg (for all gephyrin constructs) or 2 µg (for all PIAS-3 constructs) of DNA using poly-ethylamine (PEI, Polysciences Inc., 23966) according to the manufacturer recommendation. 24h later, the cells were lysed in EBC buffer (50mM Tris pH 8.0, 120mM NaCl, 0.5% NP-40) containing

proteasome inhibitor or complete-mini protease inhibitor cocktail tablets (Roche diagnostic, #11836153001) and phosphatase cocktail 2 and 3 (Sigma #P5726 and #P0044).

Transfected HEK293 cells were treated overnight with the general proteasome inhibitor: MG132 (or ZLLal) form 5μM/mL till 15μM/mL (Tocris#1748) or with DMSO (equal volume, Sigma, D2438).

Immunoprecipitation and Western Blotting

Interaction between two proteins was determined using the heterologous cells HEK293. For the immunoprecipitation (IP) followed by Western blot (WB) assays the cell lysates were incubated 90min at 4°C with 1-2μg purified antibody followed by incubation with protein A/G UltraLink Resin (Thermo Scientific, #53133) 45min at 4°C. Unspecific binding to the resin was minimized by washing with EBC based high salt buffer (50mM Tris, 500mM NaCl, 1% NP-40) followed by washes with normal EBC buffer. The samples were boiled with SDS sample buffer containing 15% fresh β-mercaptoethanol at 90°C for 4min, and separated on appropriate acrylamide % SDS gel at 140V. The proteins were transferred onto a PVDF membrane on which the Western blot could be performed. The membrane was blocked with 5% Western blocking reagent (Roche, #11921681001) then incubated with the primary antibody mixture for 3hrs or overnight. After washing with Tris-buffered saline with Tween20 (TBS-T) the membranes were incubated with the secondary antibodies mixture containing either Donkey horse radish peroxidase antibodies (HRP 1:10000, form Jackson ImmunoResearch: mouse #715-035-150 and rabbit #711-035-152) or fluorescent secondary's (1:30000): mouse IR680 (#926-68022) or rabbit IR 800 (#926-32213) from Odyssey-AB /Li-COR. For loading controls protein lysates were boiled with 5x SDS buffer before performing Western blot with the appropriate antibodies.

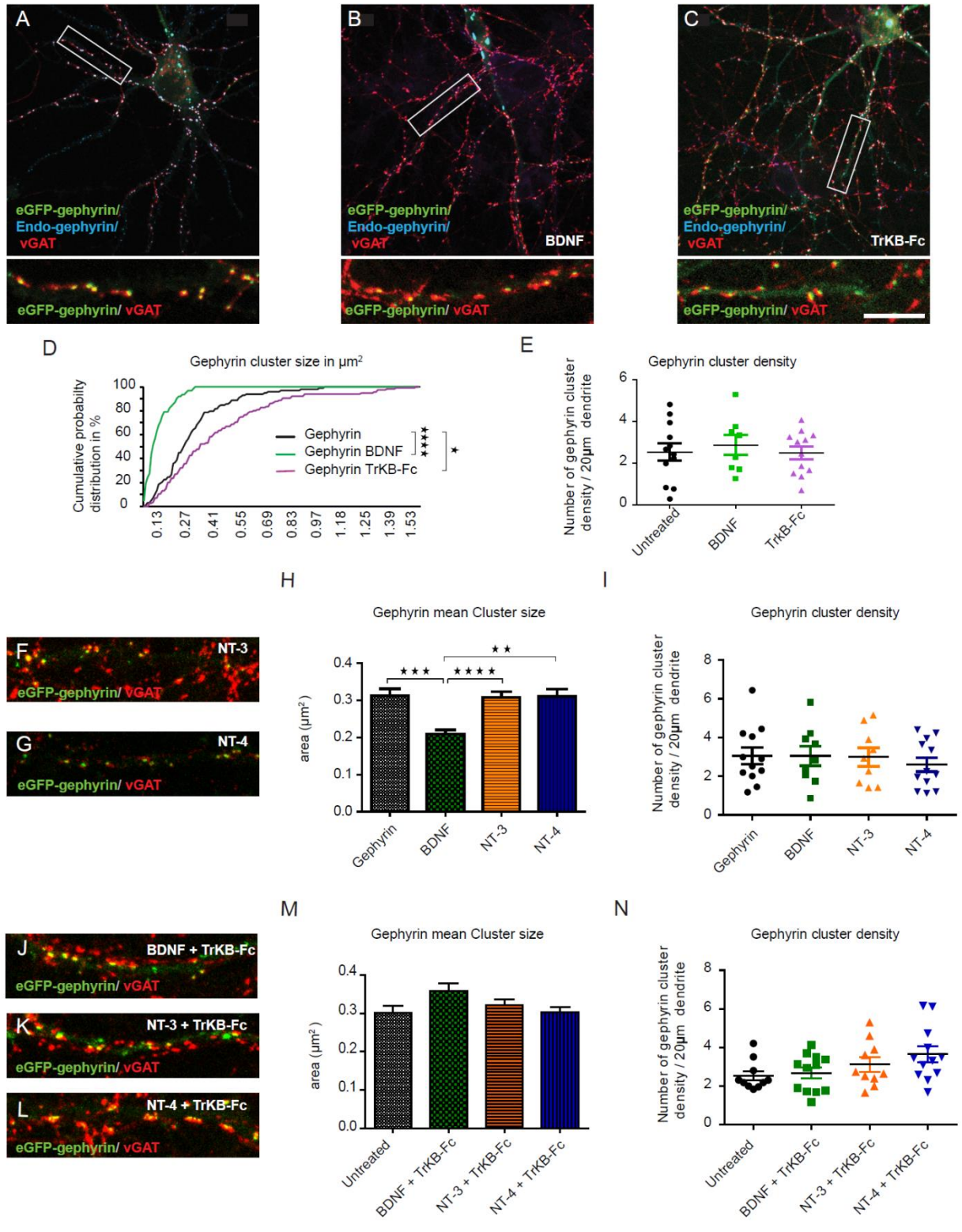
Antibodies: Mouse anti-Myc tag (1:5000, Roche #11667149001), rabbit anti-Myc tag (1:1000, Cell Signaling #2278S), mouse anti-FLAG tag (1:5000, Sigma Aldrich #F3165), rat anti-FLAG tag (1:3000, Sigma Aldrich #SAB4200071).

Results

BDNF plays an important role in regulating synapse formation and function during and after development of the CNS. However, the mechanistic basis for BDNF induced change at GABAergic synapse is poorly understood. Recent reports have shown that main scaffolding protein at GABAergic synapse, gephyrin, is regulated by phosphorylation at S268 and S270 residues via ERK1/2 and GSK3β kinases [93, 146]. TrkB receptor is known to activate ERK and GSK3β pathways downstream of BDNF signaling. Hence, we rationalized that gephyrin could be a novel candidate for facilitating BDNF induced plasticity changes at GABAergic synapse. In addition to phosphorylation, cellular pathways such as SUMOylation and acetylation also converge on to gephyrin to regulate its scaffolding properties. It is likely that BDNF signaling also influences pathways other than ERK and GSK3β.

BDNF down-regulates gephyrin clustering in hippocampal neurons

In order to better characterize BDNF signaling induced change at GABAergic synapse we used eGFP-gephyrin transfected primary hippocampal neurons after 8 days *in vitro* and stained for specific markers 7 days later (8+7 DIV) (Fig. 1A). Confocal microscopy confirmed structural changes at GABAergic postsynapse in response to acute BDNF application (10ng, 90min) and/or BDNF scavenging (TrkB-Fc). Consistent with published literature [102, 108], acute application of BDNF was sufficient to downregulate eGFP-gephyrin from GABAergic synapses. Quantification for change in cluster area showed a significant reduction after BDNF treatment (Fig. 1B, D; 0.15μm²±0.009 versus 0.29μm²± 0.019, KS test, $P<0.0001$) compared to untreated eGFP-gephyrin. However, eGFP-gephyrin cluster density was not affected after BDNF treatment (Fig.1E; 2.85 ± 0.47 versus 2.52±0.4 clusters /20μm; Two-tailed Student t-test, $P=0.73$).



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Scavenging BDNF using a chimera of TrkB extracellular domain conjugated to IgG fragment (TrkB-Fc) allowed us to study the effect of blocking BDNF signaling on eGFP-gephyrin scaffolding at GABAergic synapses. We treated transfected neurons with TrKB-Fc (1µg/mL, 90min) and noticed a pronounced increase in eGFP-gephyrin cluster size (Fig.1C-D; 0.47µm²±0.318 versus 0.29µm²± 0.19, KS test P<0.0001). We did not notice any changes in cluster density (2.47±0.30 versus 2.52±0.4 clusters /20µm; Two-tailed Student t-test P=0.93). This suggested to us that scavenging BDNF strengthens GABAergic synapses, while activating BDNF signaling weakens GABAergic synapses. Nevertheless, TrKB can also be activated by ligands such as NT-3 and NT-4, and both these neurotrophins are implicated in GABAergic synapse formation and plasticity during development [97, 267-269]. In order to study the relevance of these neurotrophins for gephyrin-induced change at GABAergic synapses, we treated eGFP-gephyrin transfected neurons with NT-3 (Fig. 1F; 10ng/mL for 90min) or NT-4 (Fig. 1G; 10ng/mL for 90min). Quantitative analyses revealed that eGFP-gephyrin cluster size is unaffected by NT-3 or NT-4 application (Fig. 1H; 0.31µm²±0.02 versus 0.31µm²±0.016 or 0.32µm²±0.020; One-Way ANOVA, $F_{(3,\ 640)}$=5.19; P=0.0015). In addition, both NT-3 or NT-4 also did not affect eGFP-gephyrin cluster density (Fig.1I; One-Way ANOVA, $F_{(3,\ 38)}$=0.27; P=0.84).

Figure 1: BDNF down-regulates gephyrin clustering in hippocampal neurons.

(A-C) Hippocampal neurons transfected (8+7) with eGFP-gephyrin treated with either BDNF (10ng/mL; 90min) or TrKB-Fc (1µg/mL; 90min) and stained for endogenous gephyrin (blue), vGAT (red). Bottom panels' show magnified dendritic segment; eGFP-gephyrin apposed to vGAT terminals were considered to be synaptic. **(D)** Cumulative probability distribution of eGFP-gephyrin cluster size, ****P<0.0001, *P<0.05 KS test. **(E)** Quantification of mean synaptic eGFP-gephyrin cluster density per 20µm dendrite. **(F-G)** Representative dendritic sections of hippocampal neurons transfected (8+7) with eGFP-gephyrin treated with different neurotrophins (10ng/mL; 90min) such as NT-3 or NT-4. **(H)** Quantification of mean synaptic eGFP-gephyrin cluster size, ****P<0.0001, ***P<0.005, **P<0.01; KS test. **(I)** Quantification of mean synaptic eGFP-gephyrin cluster density per 20µm dendrite. **(J-L)** Representative dendritic sections of hippocampal neurons transfected (8+7) with eGFP-gephyrin pretreated with TrKB-Fc (1µg/mL; 90min) prior to treatment with BDNF, NT-3 or NT-4 (10ng/mL; 90min). **(M-N)** Quantification of mean synaptic eGFP-gephyrin cluster size and cluster density per 20µm dendrite. Scale bar 10µm.

We next explored the mechanistic basis for TrKB-Fc mediated changes in eGFP-gephyrin clustering changes at GABAergic synapse. For this, we treated cultured primary neurons with BDNF, NT-3, NT-4 along with TrkB-Fc and observed for changes in eGFP-gephyrin morphology. Quantification for eGFP-gephyrin cluster size confirmed that the action of BDNF can be blocked effectively via TrKB-Fc (Fig. 1J, M; 0.35μm²±0.02 versus 0.30μm²±0.018, KS test P=0.08). Furthermore, NT-3 or NT-4 treatments in the presence of TrKB-Fc did not alter eGFP-gephyrin cluster size (Fig. 1K-M; One-Way ANOVA, $F_{(3, 792)}$=2.4; P=0.065). None of the BDNF, NT-3 or NT-4 treatments affected the eGFP-gephyrin cluster density (Fig. 1J-L, N; One-Way ANOVA, $F_{(3, 40)}$=2.33; P=0.09). These results show that BDNF specifically acts on gephyrin to modulate its scaffolding at GABAergic postsynapse.

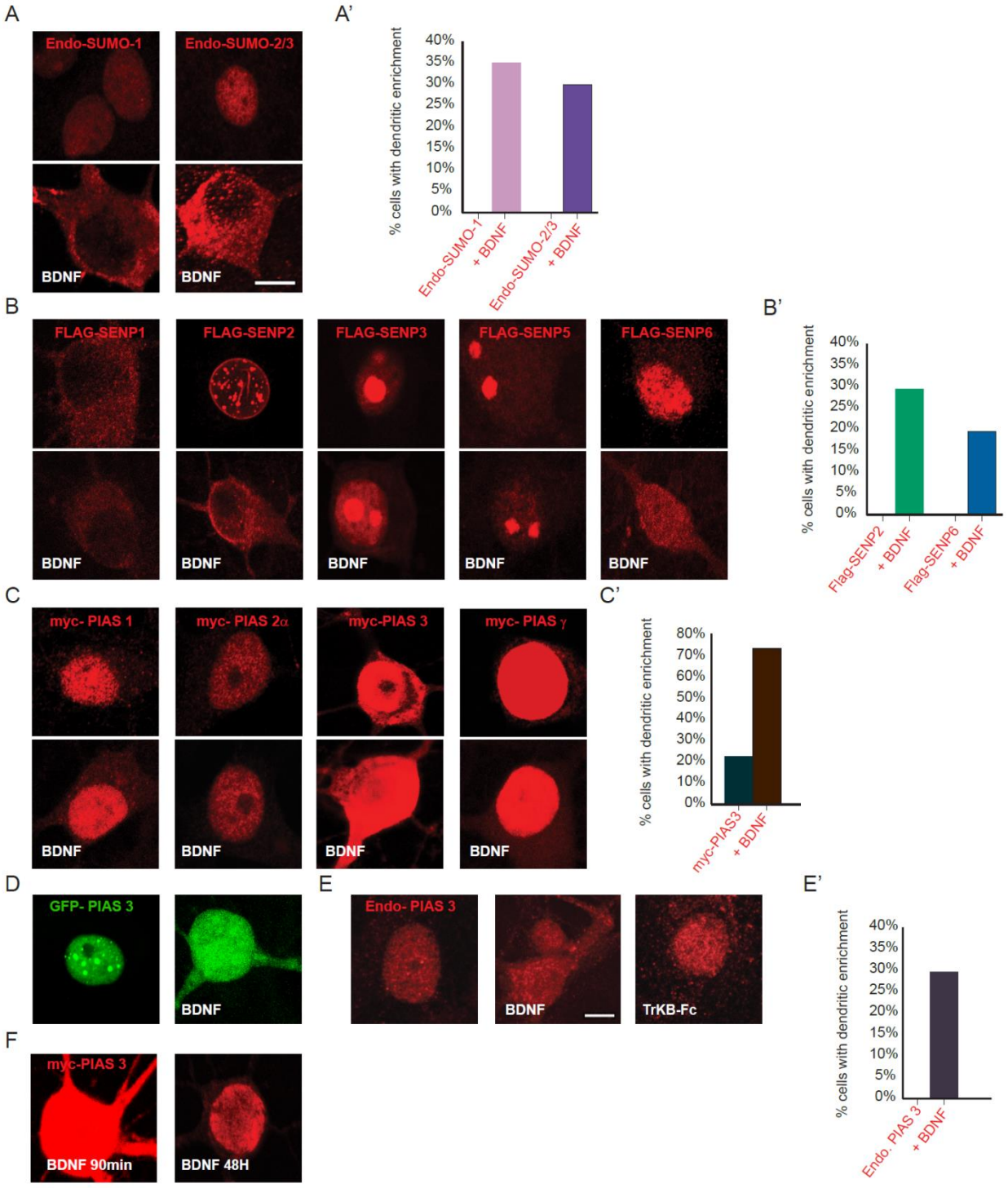
Acute BDNF treatment affects proteins of the SUMO pathway

Diverse signaling pathways converge onto gephyrin scaffold and modify specific residue to regulate its scaffold size and density [18]. Of the phosphorylation, acetylation and SUMOylation signaling cascades converging on to the gephyrin scaffold, SUMOylation seems to act upstream of the phosphorylation and acetylation.

Hence, we tested the direct role of BDNF signaling in the modulation of SUMO pathway. For this, we either stained directly for the endogenous SUMO (SUMO-1, -2/3) or stained for the myc-PIAS (myc-PIAS-1, -2, -3, -4), FLAG-SENP (SENP-1, -2, -3, -5, -6) either in the presence or absence of BDNF (10ng, 90min). The application of BDNF induced SUMO-1, SUMO-2/3, SENP-2, SENP-6 and PIAS-3 to translocate from the nucleus to soma and dendrites (Fig. 2A-E). To confirm the specificity of BDNF signaling in the translocation of specific proteins within the SUMO pathway, we tested the response of endogenous SUMO-1, SUMO-2/3 proteins to BDNF using two different antibodies, and found similar subcellular localization change in response to BDNF application (Fig. 2A).

Only PIAS-3 responded to BDNF treatment; hence, we tested two different N-terminal tags on PIAS-3 to eliminate the possibility for experimental artefacts (Fig. 2C-D). Both our myc-PIAS-3

and eGFP-PIAS-3 proteins tested in primary neurons showed a clear dendritic enrichment after BDNF application. We next tested the endogenous PIAS-3 and found a similar subcellular localization change in the presence of BDNF (Fig. 2E). Interestingly, myc-PIAS-3 dendritic translocation is reversed 48hrs post BDNF application (Fig. 2F), showing that BDNF signaling and protein SUMOylation are dynamic. To test this idea, we examined resting state neurons for subcellular localization differences in myc-PIAS-3, and quantification showed us that around 20% of PIAS-3 transfected neurons had both nuclear and dendritic distribution. However, after BDNF application, the number of transfected neurons with dendritic enrichment of PIAS-3 significantly increased to 70% (Fig. 2C'). Our observations so far suggest that BDNF signal upstream of the SUMO pathway influencing PIAS-3 subcellular localization.



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The SUMO-deficient gephyrin mutants are insensitive to BDNF

Gephyrin is a SUMO substrate and can directly interact with PIAS-3; hence, it is likely that BDNF influences changes at GABAergic synapse via gephyrin SUMOylation. The knowledge that gephyrin is SUMO-1 modified at K148 and SUMO-2 modified at K724 sites allows us to test this hypothesis directly. Hence, we transfected gephyrin SUMO-deficient mutants: eGFP-K148R and eGFP-K724R into primary hippocampal neurons and analyzed for changes in gephyrin cluster size and density in the presence or absence of BDNF (90min; short term) (Fig. 3A-E).

In absence of BDNF, quantification for cluster sizes of eGFP-K148R mutant showed a significant increase in the size compared to eGFP-gephyrin control (Fig. 3B, G; 0.42μm²±0.024 versus 0.31μm²±0.02, KS test P=0.025). However, eGFP-K724R mutant only showed a tendency towards significantly bigger cluster size (Fig. 3C, G; 0.40μm²±0.02 versus 0.31μm²±0.02, KS test P=0.187). In condition of BDNF, quantification for cluster sizes showed that both SUMO-1 and -2 mutants of gephyrin are insensitive to BDNF treatment (Fig. 3F; 0.42μm²±0.024 versus 0.41μm²±0.02; and 0.40μm²±0.02 versus 0.35μm²±0.02; KS test P=0.606). Moreover, quantification for cluster density showed no change in density for eGFP-K148R and eGFP-K724R SUMOs mutants before (Fig. 3G; One-Way ANOVA $F_{(2,\ 33)}$=1.11; P=0.34) and after BDNF treatment (Fig. 3G; One-Way ANOVA $F_{(4,\ 53)}$=2.33; P=0.067).

These results confirm that BDNF facilitates plasticity change at GABAergic synapse via the regulation of gephyrin SUMOylation at K148 and K724 respectively.

Figure 2: BDNF alters the specific sub-cellular localisation of specific proteins of the SUMO pathway.

(A-C) Primary hippocampal neurons (8+7 DIV) stained for endogenous SUMO, FLAG-SENP, or myc-PIAS either with or without BDNF (10ng/mL; 90min). **(D)** eGFP-PIAS-3 transfected neurons showing dendritic enrichment after BDNF (10ng/mL; 90min) treatment. **(E)** Endogenous PIAS-3 in primary hippocampal neurons with or without BDNF (10ng/mL; 90min) or TrKB-Fc (1μg/mL; 90min) treatments. **(A'-E')** Quantification of neurons showing subcellular localisation changes after BDNF (10ng/mL; 90min) treatment. **(F)** Subcellular localisation of myc-PIAS-3 in neurons is reversed 48hr post BDNF (10ng/mL) treatment. Scale bar 5μm.

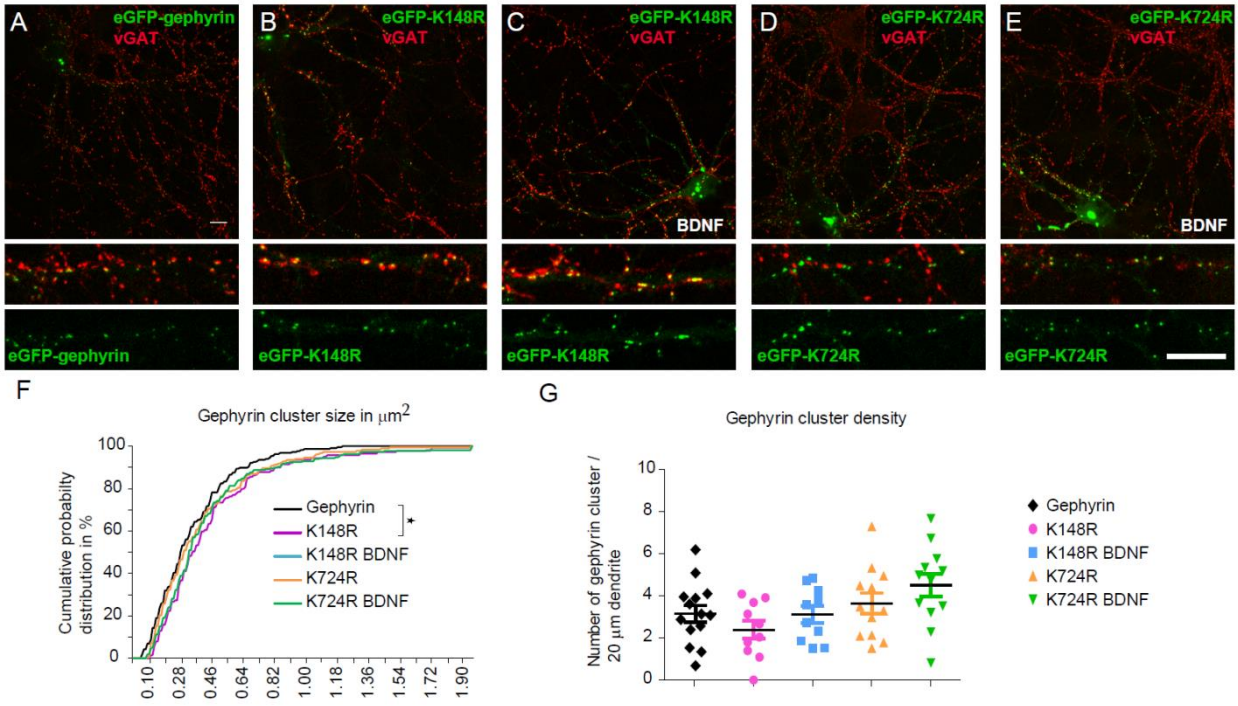


Figure 3: Gephyrin SUMO-1 and SUMO-2 site mutations are insensitive to BDNF.

(A-E) Representative images of DIV (8+7) neurons expressing either eGFP-gephyrin, or eGFP-K148R or eGFP-K724R in absence of BDNF **(A, B, D)** and after BDNF treatment **(C, E).** GABAergic terminals were identified by staining for the presynaptic marker vGAT (red). Synaptic eGFP-gephyrin clusters were analyzed based on their apposition to vGAT positive terminals. **(F)** Cumulative probability distribution of eGFP-gephyrin cluster size, **P*<0.05 KS test. **(G)** Quantification of mean synaptic eGFP-gephyrin cluster density per 20μm dendrite showing no differences between untreated and BDNF treated neurons. Scale bar 10μm.

BDNF regulates PIAS-3 function for gephyrin clustering change

Substrate SUMOylation is facilitated by the presence of SUMO E3 ligase [195, 220, 270]. PIAS-3 is an E3 ligase identified to influence gephyrin SUMO conjugation [Ghosh et al., submitted]. Given our observation that BDNF signaling influences PIAS-3 subcellular localization, we wondered if BDNF also influenced PIAS-3 function in neurons. In order to test this idea we co-transfected myc-PIAS-3 with eGFP-gephyrin into DIV (8+7) neurons and assessed for myc-

PIAS-3 function by looking at morphological changes in gephyrin clustering, in the presence or absence of BDNF (Fig. 4B-C).

Neurons transfected with myc-PIAS-3 showed a significant increase in cluster size in comparison to eGFP-gephyrin alone control (0.79μm²±0.07 versus 0.33μm²±0.02; KS test $P<0.0001$). Moreover, in the presence of myc-PIAS-3 eGFP-gephyrin cluster density was also significantly reduced (Fig. 4B, H, I; 1.09±0.12 versus 2.74±0.46 clusters /20μm; Two-tailed Student' T-test $P=0.005$). Addition of 10ng/mL BDNF for 90 min brought eGFP-gephyrin cluster size to base line level, suggesting BDNF influences PIAS-3 function (Fig. 4C; 0.4μm²±0.04 versus 0.79μm²±0.07, KS test $P=0.006$). However, we did not observe any significant difference in eGFP-gephyrin cluster density when myc-PIAS-3 co-transfected neurons were treated with BDNF (Fig. 4I; 1.4±0.12 versus 1.09±0.12 clusters /20μm; Two-tailed Student' T-test $P=0.14$). These results suggest that BDNF down-regulates eGFP-gephyrin clustering size via PIAS-3 function.

PIAS-3 belongs to a larger family of PIAS proteins and was first characterized as regulator of transcriptional activity of STAT3 proteins [216, 271]. Subsequently, it was demonstrated that PIAS-3 is also a SUMO E3 ligase [195]. PIAS family of proteins share 5 conserved domains, among which the SP-Ring domain is important for the SUMO E3 ligase function [195, 209]. In order to study the importance of PIAS-3 E3 ligase activity for eGFP-gephyrin clustering, we co-transfected the SP-Ring domain mutant of PIAS-3 (myc-PIAS-3Rm), into neurons, along with eGFP-gephyrin. Interestingly, inactivation of the SUMO E3 ligase activity of PIAS-3 (myc-PIAS-3Rm) still showed an increase in eGFP-gephyrin cluster size (Fig. 4E, H; 0.55μm²±0.05 versus 0.33μm²±0.02, KS test $P=0.002$) and a decrease in density (Fig. 4F, I; 1.1±0.22 versus 2.74±0.46 clusters/20μm; Two-tailed Student' T-test, $P=0.008$), similar to myc-PIAS-3.

The increase in eGFP-gephyrin cluster size in the presence of myc-PIAS-3Rm although significantly larger than eGFP-gephyrin alone, was still smaller than in neurons co-expressing myc-PIAS-3 (KS test $P=0.037$). This suggested us that myc-PIAS-3 and myc-PIAS-3Rm influence gephyrin clustering perhaps via two independent cellular mechanisms. In order to test this idea, we treated neurons co-transfected with myc-PIAS-3Rm and eGFP-gephyrin with BDNF and found that BDNF treatment had no effect on myc-PIAS-3Rm induced increase in eGFP-

gephyrin cluster size (0.56µm²±0.04 versus 0.55µm²±0.05, KS test *P*=0.54). This implicated BDNF signaling for PIAS-3 SP-Ring domain function in gephyrin cluster size regulation.

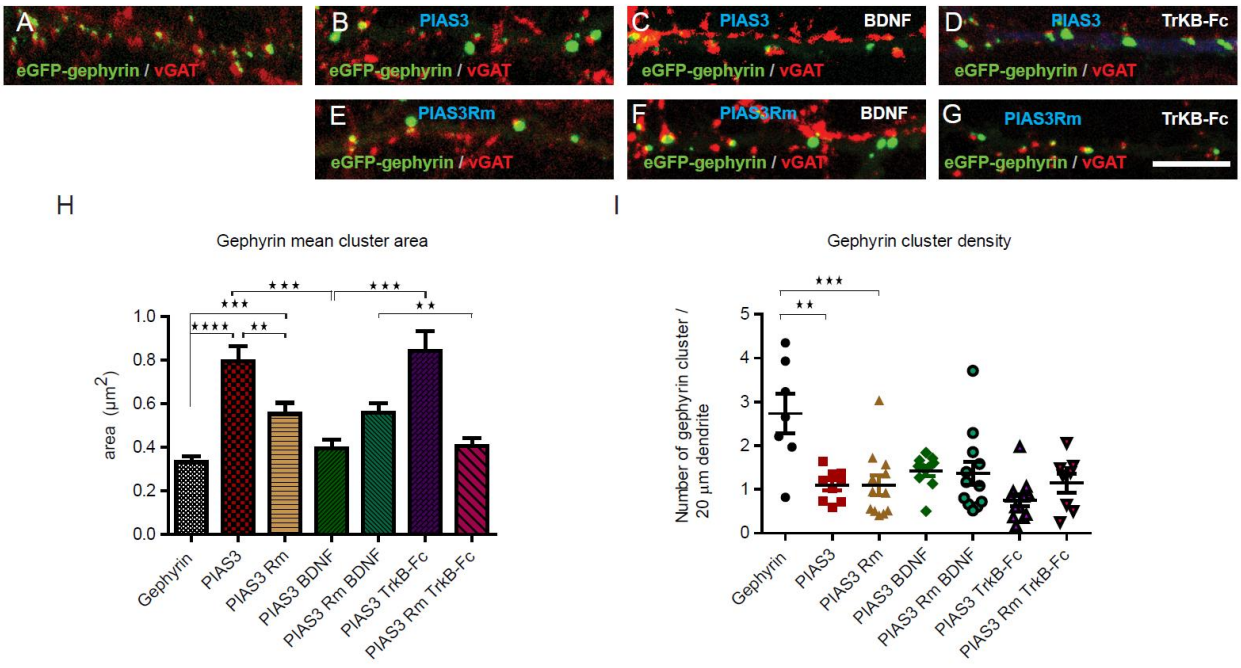


Figure 4: BDNF regulates PIAS-3 function for gephyrin cluster change.

(A-G) Morphology of DIV (8+7) neurons co-transfected with eGFP-gephyrin alone, or with either myc-PIAS-3, or myc-PIAS-3Rm, with or without BDNF (10ng/mL; 90min) or TrKB-Fc (1µg/mL; 90min) treatment. eGFP-gephyrin (green) clusters synaptic localization was determined based on their apposition to vGAT positive terminal (red). **(H)** Quantification of eGFP-gephyrin cluster size shows an increase with the co-expression of either myc-PIAS-3 or myc-PIAS-3Rm, ****P<0.0001, ***P<0.001, **P<0.01 KS test from the cumulative. **(I)** Quantifications of synaptic eGFP-gephyrin cluster density per 20µm dendritic length shows a significant decrease in the presence of myc-PIAS-3 or myc-PIAS-3Rm, ***P<0.001 One-Way ANOVA Kruskal Wallis test. Scale bar 10µm.

If BDNF signaling is an important regulator of PIAS-3 SP-Ring domain, we wondered whether suppression of BDNF signaling would influence the SP-Ring domain independent function of PIAS-3 for eGFP-gephyrin cluster regulation. Hence, to sequester endogenous BDNF signaling we used TrKB-Fc. Neurons co-transfected with myc-PIAS-3 or myc-PIAS-3Rm along with eGFP-gephyrin were treated with TrKB-Fc and analyzed for change in eGFP-gephyrin clusters at vGAT positive terminals (Fig. 4D, G). Cells transfected with myc-PIAS-3 exhibited a similar phenotype of larger eGFP-gephyrin clusters in the presence of TrkB-Fc (Fig. 4D; 0.84μm²±0.09 versus 0.79μm²±0.07, KS test *P*=0.84). When we compared changes in neurons expressing myc-PIAS-3Rm in presence of TrkB-Fc, we could see a significant decrease in size compare to BDNF treated neurons (Fig. 4H; 0.41μm²±0.03 versus 0.56μm²±0.04, KS test *P*=0.03). Quantification for eGFP-gephyrin cluster density showed no change in myc-PIAS-3 or myc-PIAS-3Rm co-expressing neurons (Fig. 4I; One-Way ANOVA Kruskal Wallis test, *P*=0.56). This confirms our idea that blocking BDNF signaling activates an alternative pathway to influence SP-Ring domain independent function of PIAS-3 for gephyrin cluster size regulation.

PIAS-3 SP-Ring domain-independent regulation of gephyrin is via C-terminus sequence

Our result so far identifies a PIAS-3 SP-Ring-dependent and independent function that is influenced by BDNF signaling. To understand the mechanistic basis of SP-Ring domain-independent effect on eGFP-gephyrin clustering, we used different truncation mutations of myc-PIAS-3 (Fig. 5A) and co-expressed each of them with eGFP-gephyrin. Analysis of eGFP-gephyrin cluster size and density allowed us to determine the role of specific PIAS-3 domains (Fig. 5B-G).

Consistent with our earlier observations, co-expression of myc-PIAS-3 led to an overall increase in eGFP-gephyrin cluster size (Fig. 5B). Our observations were confirmed by quantification (Fig. 5H; 0.68μm²±0.06 versus 0.27μm²±0.02; KS test *P*<0.0001). In addition, we also saw a significant decrease in cluster density in neurons expressing myc-PIAS-3 (Fig. 5I; 1.09±0.12 versus 2.8±0.29 clusters/20μm; Two-tailed Student' T-test *P*=0.0007). Co-expression of myc-PIAS-3 N-terminus (1-273AA), containing the SAP and PINIT domains did not cause any

increase in eGFP-gephyrin cluster size compared to eGFP-gephyrin alone control neurons (Fig. 5C, H; 0.36µm²±0.02 versus 0.27µm²±0.02; KS test P=0.006) or to myc-PIAS-3 (KS test P<0.0001).

Consistent with these observations we also did not see any reduction of eGFP-gephyrin density in neurons co-expressing the myc-PIAS-3 (1-273) (Fig. 5I; 3.22±0.52 versus 1.09 ±0.12versus 2.8±0.29 clusters/20µm; One-way ANOVA, $F_{(2, 27)}$=7.89, P=0.002).

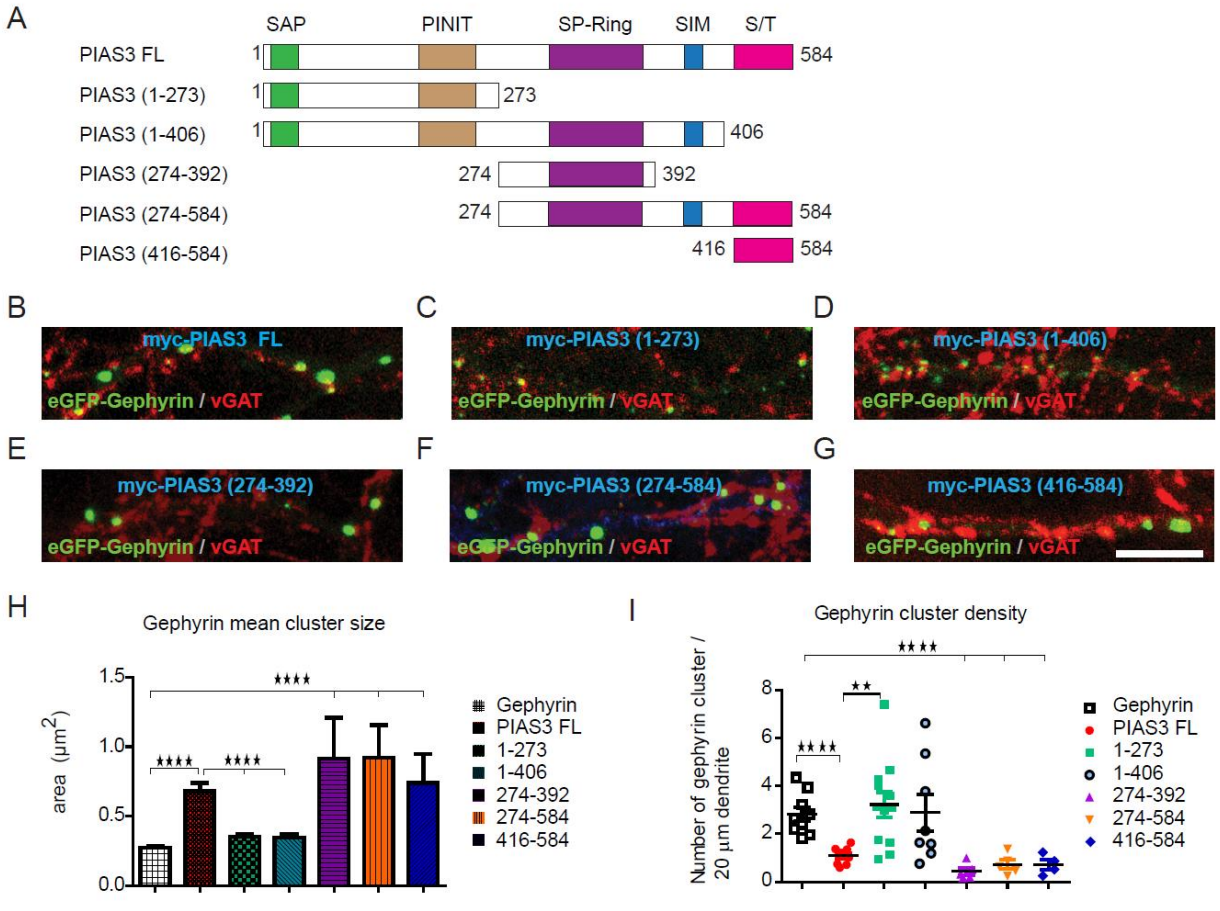


Figure 5: PIAS-3 SP-Ring domain independent regulation of gephyrin is via C-terminus sequence

(A) Cartoon of various truncated myc-PIAS-3 used in the current study. **(B-G)** Morphology of DIV (8+7) neurons co-transfected with eGFP-gephyrin and one of the myc-PIAS-3 truncation mutants. **(H)** Quantification of synaptic eGFP-gephyrin cluster size, ****P<0.0001, KS test. **(I)** Quantifications of synaptic eGFP-gephyrin cluster density per 20µm dendritic length, ****P<0.0001 One-Way ANOVA followed by a Tukey test. Scale bar 10µm

When we compared the central fragment containing the SP-Ring domain myc-PIAS-3(273-392) we found a significant increase in eGFP-gephyrin cluster size compared to eGFP-gephyrin alone control (Fig. 5E, H; 0.9μm²±0.3 versus 0.27μm²±0.02; KS test $P<0.0001$), comparable to that seen in neurons co-expressing myc-PIAS-3 (KS test $P=0.22$). Similarly, we found a significant reduction of eGFP-gephyrin cluster density in neurons co-expressing myc-PIAS-3(273-392) (Fig. 5I; 0.43±0.15 versus 2.8±0.29 clusters/20μm; Two-tailed Student' T-test $P=0.01$).

Next, we tested two different myc-PIAS-3 C-terminus truncations; myc-PIAS-3 (274-584) and myc-PIAS-3(416-584). The co-expression of either of these two C-terminus fragment of PIAS-3 significantly increased the eGFP-gephyrin cluster size (Fig. 5H; 0.9μm²±0.2 versus 0.27μm²±0.02 and 0.74μm²±0.2 versus 0.27μm²±0.02; One-way ANOVA $F_{(3,\ 23)}=20.89$, $P<0.0001$). In addition, we also saw a significant reduction of eGFP-gephyrin cluster density in neurons co-expressing either myc-PIAS-3 (274-584) or myc-PIAS-3(416-584) along with eGFP-gephyrin (Fig. 5F-I; One-way ANOVA $P<0.0001$). These results identify PIAS-3 C-terminus between amino acids 416-584 as crucial determinant for SP-Ring domain independent regulation of eGFP-gephyrin clustering in neurons

When we tested the neurons that co-expressed the N-terminus PINIT domain along with the central SP-Ring domain, myc-PIAS-3(1-406), the eGFP-gephyrin clusters did not grow in size (Fig. 5D, H; 0.27μm²±0.02 versus 0.34μm²±0.03 and 0.68μm²±0.06; One-Way ANOVA, Kruskal-Wallis test $P<0.0001$). Analysis for change in eGFP-gephyrin cluster density also did not show any significant reduction (Fig. 5I; 2.74±0.46 versus 2.89±0.76 and 1.09±0.12 clusters/20μm; One-Way ANOVA $F_{(2,\ 23)}=5.345$, $P=0.012$). These results suggest to us that N-terminus PINIT domain can suppress the SP-Ring domain function in the absence of the C-terminus sequence.

BDNF affects both PIAS-3 SP-Ring domain and C-terminus function

Given our observation that PIAS-3 SP-Ring domain (273-392AA) and C-terminus sequence (416-584AA) produce a similar morphological change in gephyrin clustering, we wondered whether BDNF signaling influenced both these phenotypes, albeit via distinct mechanisms. In order to test this idea, we co-transfected neurons with eGFP-gephyrin and myc-PIAS-3(273-392) and treated them with BDNF. The treatment of neurons co-expressing myc-PIAS-3(273-392) was sufficient to abrogate the SP-Ring domain activity as eGFP-gephyrin cluster size returned to base line levels (Fig. 6A, B; 0.26μm²±0.02 versus 0.9μm²±0.3; KS test *P*=0.006). The eGFP-gephyrin cluster density was returned to base line levels after BDNF treatment in myc-PIAS-3(273-392) expressing neurons (Fig. 6A, C; 3.25±0.6 versus 0.73±0.19 clusters/20μm; Two tailed Student' T-test, *P*=0.004). This demonstrates the importance of BDNF signaling in regulating SP-Ring domain function of PIAS-3.

Our earlier results show that myc-PIAS-3Rm is sensitive to BDNF scavenging via TrKB-Fc treatment. Hence, it is possible that myc-PIAS-3Rm effect on eGFP-gephyrin clustering is via its C-terminus sequence. Therefore, we tested to see whether myc-PIAS-3(416-584)-mediated increase in eGFP-gephyrin clustering could be reversed by TrKB-Fc treatment. Neurons co-transfected with eGFP-gephyrin and myc-PIAS-3(416-584) were treated with either BDNF or TrKB-Fc (Fig. 5D-D´) and analyzed for change in eGFP-gephyrin cluster size and density. Neurons co-expressing myc-PIAS-3(416-584) did not respond to BDNF treatment (Fig. 6D, E; 0.6μm²±0.08 versus 0.79μm²±0.15; KS test *P*=0.097). Similarly, we did not observe any significant differences in eGFP-gephyrin cluster density after BDNF treatment (Fig. 6D, F; 1.5±0.3 versus 1±0.3 clusters/20μm; Two-tailed Student' T-test *P*=0.18). However, TrKB-Fc treatment caused a significantly reduced eGFP-gephyrin cluster size in myc-PIAS-3 (416-584) co-expressing neurons (Fig. 6D', E; 0.41μm²±0.003 versus 0.79μm²±0.15; KS test *P*=0.047). Analysis for eGFP-gephyrin cluster density also showed a significant upregulation (Fig. 6F; 3.8 ±0.5 versus 1±0.3 clusters/20μm; Two-tailed Student' T-test *P*=0.015) compare to untreated neurons.

Taken together, our results highlight the importance of BDNF signaling for PIAS-3 function and gephyrin scaffold regulation at GABAergic synapse. By identifying divergent roles in TrkB activation and inhibition for PIAS-3 function, our results uncover a novel bimodal basis for BDNF-induced plasticity at GABAergic synapses.

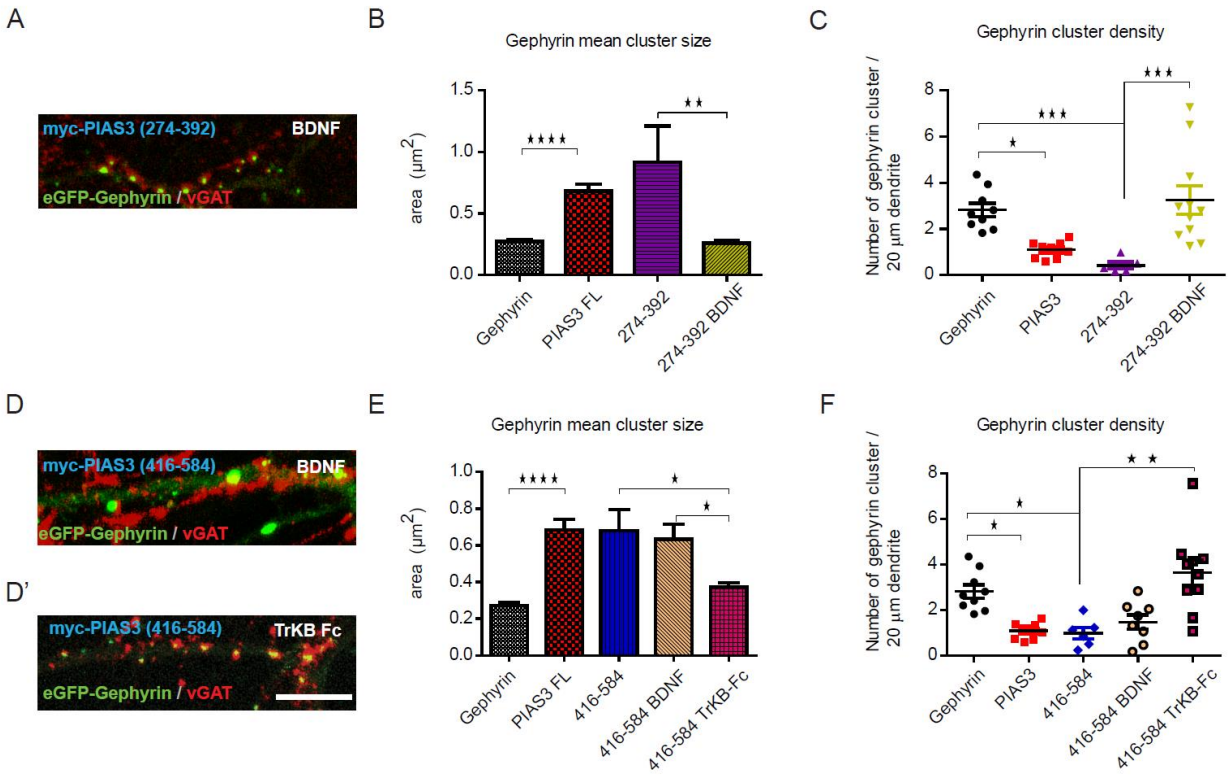


Figure 6: BDNF affects both PIAS-3 SP-Ring domain and C-terminus function.

(A) Morphology of neurons co-transfected with eGFP-gephyrin and myc-PIAS-3(273-392) and treated with BDNF (10ng/mL; 90min). **(B)** Quantification synaptic eGFP-gephyrin cluster size, ****P<0.0001, **P<0.01 KS test. **(C)** Quantifications of synaptic eGFP-gephyrin cluster density per 20µm dendritic length, ***P<0.001 One-Way ANOVA. **(D-D')** Morphological analysis of neurons co-transfected with eGFP-gephyrin and myc-PIAS-3(416-584) treated with either BDNF (10ng/mL, 90min) or TrKB-Fc (1µg/mL, 90min). **(E)** Quantification of synaptic eGFP-gephyrin cluster size, ****P<0.0001, *P<0.05 KS test. **(F)** Quantifications of synaptic eGFP-gephyrin cluster density per 20µm dendritic length, **P<0.01 One-Way ANOVA. Scale bar 10µm.

Gephyrin interacts with PIAS-3 at SP-Ring and SAP/PINIT domains

We have identified direct interaction between myc-PIAS-3 and FLAG-gephyrin using the hertrologus HEK293 cells [Ghosh et al., submitted]. However, our current studies highlight the importance of PIAS-3 SP-Ring domain and C-terminus for gephyrin cluster regulation. Hence, we wondered if PIAS-3 harbored more than one gephyrin binding site. In order to test this we co-transfected HEK293 cells with myc-PIAS-3 deletion fragments along with FLAG-gephyrin. IP for myc-PIAS variants followed by WB for FLAG-gephyrin showed gephyrin interaction with the full length PIAS-3, N-terminus PINIT (1-273AA) and SP-Ring domains (274-392AA) respectively (Fig. 7A-B, first panel). The protein loading controls are showed in the panels below (Fig. 7B, middle and lower panels).

We then examined to identify the binding site for PIAS-3 on gephyrin. In order to do this we co-transfected myc-PIAS-3 and FLAG-gephyrin, or FLAG-G, or FLAG-GC or FLAG-E domains into HEK293 cells and immunoprecipitated myc-PIAS-3, followed by WB for FLAG-gephyrin or its domains (Fig. 7C). Our biochemical analysis showed multiple interaction sites for PIAS-3 on gephyrin as we could successfully pull down FLAG-G, FLAG-GC and FLAG-E fragments along with myc-PIAS-3. We identified and characterized two SUMOylation sites on gephyrin, K148 situated in the N-terminus G-domain and K724 situated in the C-terminus E-domain. Hence, its not surprising that PIAS-3 can interact with both the G and E domains of gephyrin.

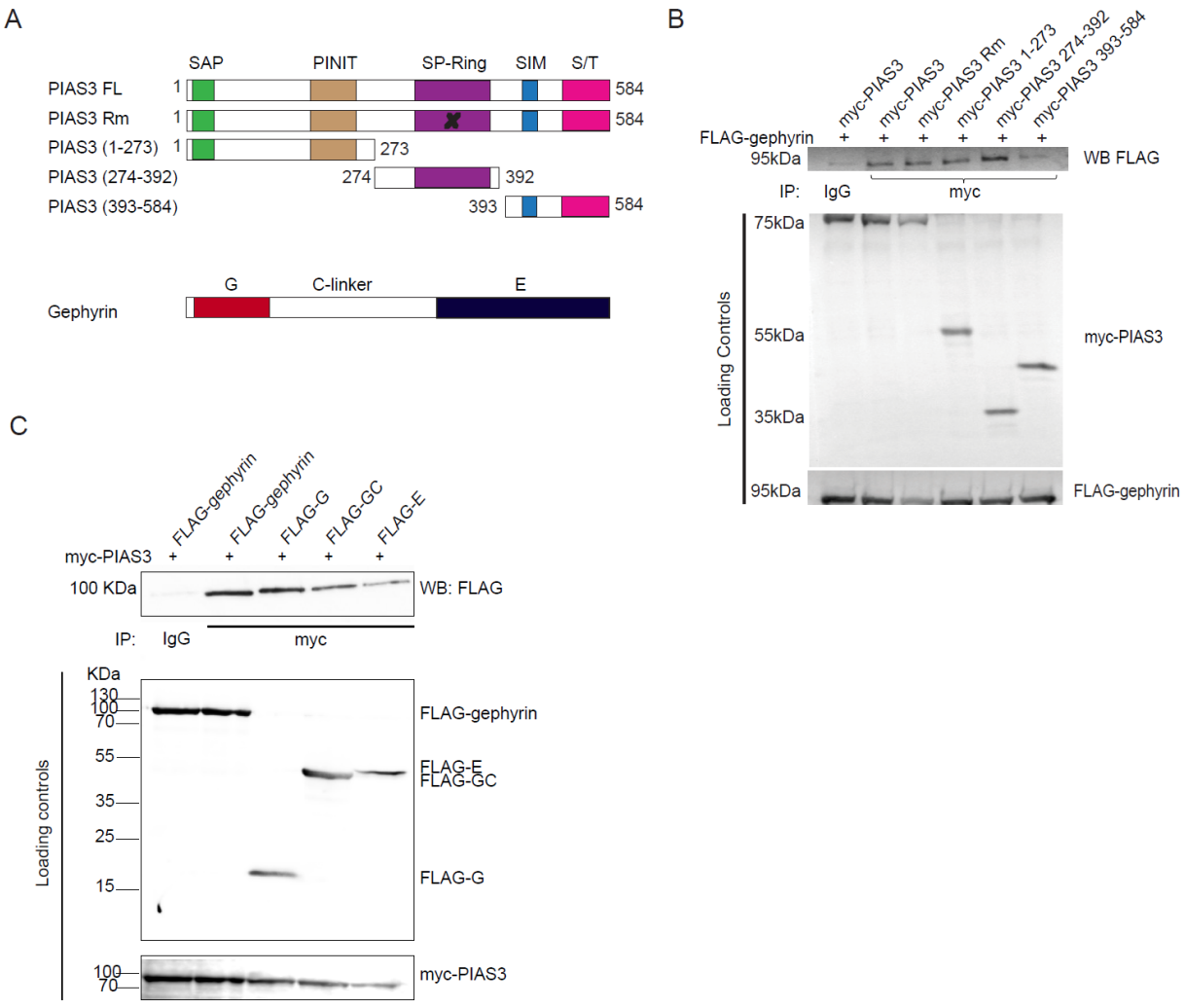


Figure 7: Gephyrin interacts with PIAS-3 at SP-Ring and SAP/PINIT domains.

(A) Cartoon of different myc-PIAS-3 truncation mutations used for biochemical analysis of gephyrin interaction in HEK293 cells. **(B)** Interaction between FLAG-gephyrin and myc-PIAS-3. IP for myc-PIAS-3 followed by WB for FLAG-gephyrin. Protein loading controls are shown in the lower panels. **(C)** Interaction between myc-PIAS-3 and different domains of FLAG-gephyrin. IP for myc-PIAS-3 followed by WB for FLAG-gephyrin. Protein loading controls are shown in the lower panels

PIAS-3 function is autoregulated by its N-Terminal domain

Our results thus far identify SP-Ring domain and C-terminus of PIAS-3 for gephyrin cluster regulation at GABAergic synapses. In addition, we identify two interaction sites for gephyrin on PIAS-3, namely the PINIT domain and the SP-Ring domain. Hence, we next wanted to understand how PINIT and SP-Ring domains contributed to the C-terminus induced gephyrin cluster regulation. To understand this better we introduced two point mutations into the PINIT domain (I200V/T201A), and in addition we also generated the PINIT and SP-Ring domain combination mutation. We looked for clustering changes after co-transfected DIV 8+7 neurons with myc-PIAS-3PINITm or myc-PIAS-3PINITm/Rm along with eGFP-gephyrin (Fig. 8A-C). Rendering the PINIT domain inactive via two point mutations blocked myc-PIAS-3 induced eGFP-gephyrin clustering changes in size (Fig. 8D; 0.44µm²±0.04 versus 0.65µm²±0.07; KS test P=0.002) and eGFP-gephyrin cluster density (Fig. 8E; 1.4±0.16 versus 1.0±0.23 clusters/20µm; Two-tailed Student' T-test P=0.18) compared to myc-PIAS-3. The co-expression of myc-PIAS-3PINIT/Rm mutant showed a significant reduction in eGFP-gephyrin cluster size (Fig. 8D; 0.21µm²±0.03versus 0.44µm²±0.04 versus 0.65µm²±0.07, One way ANOVA P<0.0001) and a significant increase in eGFP-gephyrin cluster density (Fig. 8E; 1.9±0.3 versus 1.4±0.16 versus 1.0±0.23 clusters/20µm, One way-ANOVA $F_{(2,33)}$=0.11, P=0.03) compared to myc-PIAS-3PINITm and myc-PIAS-3.

The influence of myc-PIAS-3PINIT mutation on the SP-Ring domain function was intriguing; hence, we decided to see how the PINIT domain mutation affected biochemical interaction with gephyrin. We co-transfected HEK293 cells with myc-PIAS-3, myc-PIAS-3PINITm, or myc-PIAS-3PINIT/Rm along with FLAG-gephyrin. IP for myc-PIAS-3 followed by WB for FLAG-gephyrin showed no gephyrin interaction in the presence of PINIT domain mutation (Fig. 8F, lanes 4-5). However, when we analyzed the protein loading control we noticed that myc-PIAS-3PINITm protein levels were reduced. Furthermore, we noticed an additional protein band running at reduced molecular weight (~50 kDa). In contrast, gephyrin expression was unaffected (Fig. 8G, bottom panel). This suggests to us that PINIT domain is essential for the PIAS-3 protein integrity via proper 3D conformation.

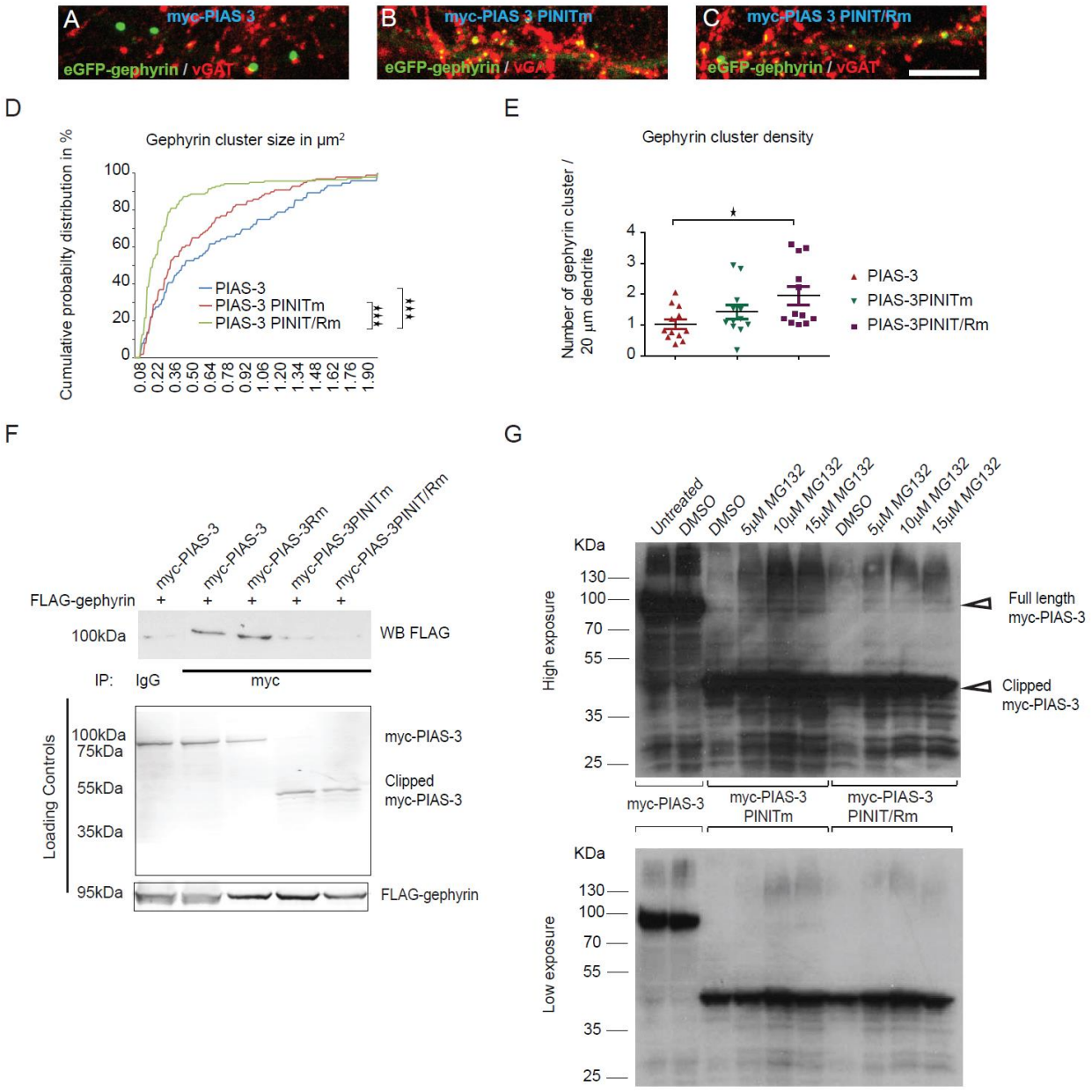


Figure 8: PIAS-3 function is autoregulated by its N-Terminal domain.

(A-C) Morphology of neurons (8+7 DIV) co-transfected with eGFP-gephyrin and myc-PIAS-3, myc-PIAS-3PINITm or myc-PIAS-3PINIT/Rm. **(D)** Cumulative probability distribution of synaptic eGFP-gephyrin cluster size, ***P<0.001, KS test. **(E)** Quantifications of synaptic eGFP-gephyrin cluster density per 20µm dendritic length. *P<0.05 One-Way ANOVA. **(F)** Interaction between FLAG-gephyrin and myc-PIAS-3, myc-PIAS-3Rm, myc-PIAS-3PINITm and myc-PIAS-3PINIT/Rm. IP for myc-PIAS-3 using anti-Myc antibody, and WB against FLAG-gephyrin. The protein loading controls are shown below. **(G)** WB for myc-PIAS-3, myc-PIAS-3PINITm and myc-PIAS-3PINIT/Rm in untreated versus MG132 treatments (5µM, 10 µM and 15 µM; 6Hrs). The lower panel shows lower exposure. Scale bar 10µm.

In order to determine the mechanistic basis for myc-PIAS-3PINITm and myc-PIAS-3PINIT/Rm degradation, we blocked 26S proteosome using the broad range inhibitior MG132. The use of MG132 at different concentration could not rescue myc-PIAS-3PINITm degradation in HEK293 cells (Fig. 8G). However, at higher exposure we could see weak bands of full length myc-PIAS-3PINITm in the presence of 10μM or 15μM of MG132 (Fig. 8G, white arrows, first panel). The mechanistic basis for PINIT mutation induced PIAS-3 proteolytic clipping and/or degradation needs further characterization.

ERK and GSK3β signaling regulate PIAS-3 activity for gephyrin clustering

It has been shown earlier that ERK1/2 and GSK3β signal transduction pathways phosphorylate gephyrin at S268 and S270 residues to modulate GABAergic transmission [93, 156]. Independent reports have shown that BDNF signaling activate ERK1/2 and GSK3β pathways downstream of TrKB [106, 272]. Furthermore, it has been shown that SUMOylation and ERK1/2 pathways cross-talk to regulate gephyrin postsynaptic clustering. Hence, we decided to further explore how BDNF induced ERK1/2 and GSK3β pathways influence PIAS-3 function.

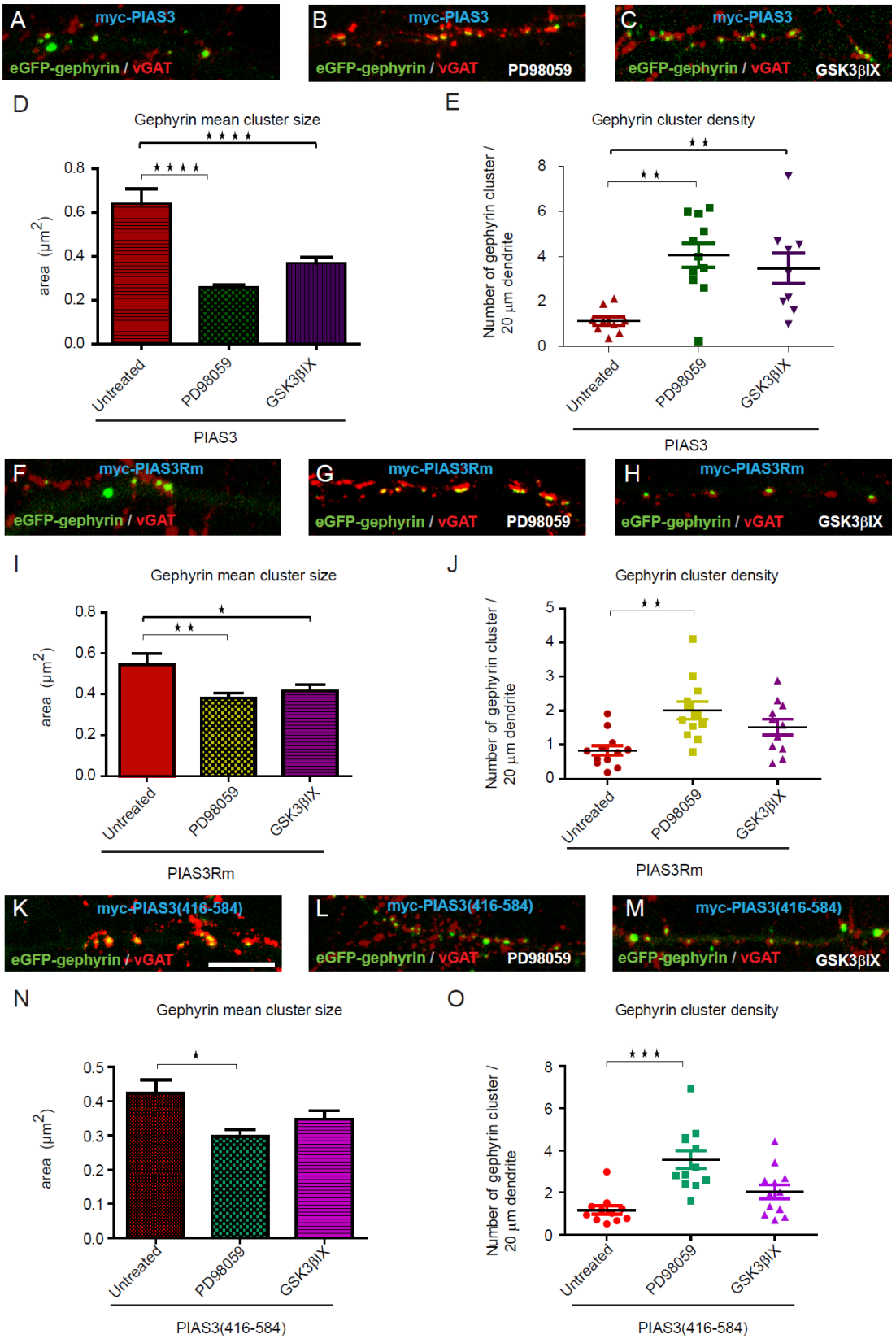
We first assesed the effect of ERK1/2 on myc-PIAS-3 modulation of eGFP-gephyrin clustering; for this, we treated neurons with the ERK1/2 inhibitor PD98059 (Fig. 9A-B). Overnight treatment of neurons with PD98059 (25μM) induced a marked decrease in gephyrin cluster size in neurons co-expressing myc-PIAS-3 (Fig. 9D; 0.26μm²±0.01 versus 0.64μm²±0.068; KS test $P<0.0001$). Furthermore, PD98059 treatment also caused a significant increase in eGFP-gephyrin cluster density in presence of myc-PIAS-3 (Fig. 9E; 4.05±0.5 versus 1.14±0.19 clusters/20μm; Two tailed Student' T-test $P<0.0001$). These results indicate that inhibition of ERK1/2 signaling blocks PIAS-3 action on eGFP-gephyrin cluster size and density.

In an attempt to define more precisely the ERK1/2 modulation domain on PIAS-3, we co-transfected neurons with eGFP-gephyrin and myc-PIAS-3Rm (Fig. 9F) or myc-PIAS-3(416-584) (Fig. 9K) and treated the neurons with PD98059 (Fig. 9G-L). ERK1/2 inhibition significantly decreased eGFP-gephyrin clustering size in neurons co-expressing either myc-PIAS-3Rm (Fig.

9I; 0.38µm²±0.024 versus 0.54µm²±0.056; KS test *P*=0.009) or myc-PIAS-3(416-584) (Fig. 9N; 0.3µm²±0.02 vs untreated 0.42µm²±0.04; KS test *P*=0.021). In addition, treating neurons with PD98059 increased eGFP-gephyrin cluster density in the presence of myc-PIAS-3Rm (Fig. 9J; 2.01±0.26 versus 0.83± 0.14 clusters/20µm; Two tailed Student' T-test *P*<0.0001) or myc-PIAS-3(416-584) (Fig. 9O; 3.5±0.43 versus 1.17±0.2 clusters/20µm; Two tailed Student' T-test *P*=0.0006). These results suggest a role for ERK1/2 phosphorylation of PIAS-3 C-terminus to influence gephyrin cluster regulation in a SUMO-independent mechansim.

Next, we investigated the effect of GSK3β on myc-PIAS-3 for gephyrin clustering. For this we used the GSK3β inhibitor, GSK3βIX (5µM), overnight and stained for morphological change in postsynaptic eGFP-gephyrin clustering (Fig. 9A, C). GSK3βIX treatment significantly decreased eGFP-gephyrin cluster size (Fig. 9D; 0.37µm²±0.025 versus 0.64µm²±0.068; KS test *P*<0.0001). Further, when we analyzed for change in eGFP-gephyrin cluster density we found that GSK3βIX treatment significantly increased the density (Fig. 9E, 3.5±0.7 versus 1.14± 0.19 clusters/20µm; Two tailed Student' T-test *P*=0.0076). These effects are similar to those caused by ERK1/2 inhibition.

Then, we examined the effect of GSK3βIX treatment in neurons co-expressing either myc-PIAS-3Rm or myc-PIAS-3(416-584) along with eGFP-gephyrin. Interestingly, GSK3β inhibition reduced eGFP-gephyrin cluster size in the presence of myc-PIAS-3Rm (Fig. 9F, H, I; 0.42µm²±0.03versus 0.54µm²±0.056; KS test *P*=0.033), again like seen upon ERK1/2 inhibition. However, when we analyzed for change in eGFP-gephyrin cluster density, we did not find any significant changes after GSK3β inhibition (Fig. 9J; 1.52±0.23 versus 0.83± 0.14 clusters/20µm; Two tailed Student' T-test *P*=0.073). In neurons co-expressing myc-PIAS-3(416-584) with or without GSK3βIX (Fig. 9K, M), we did not observe any significant change in cluster size (Fig. 9N; 0.35µm²±0.03 versus 0.42µm²±0.04; KS test *P*=0.082) or cluster density (Fig. 9O; 2.04±0.33 clusters/20µm versus 1.17±0.2; Two-tailed Mann Whitney test *P*=0.052).



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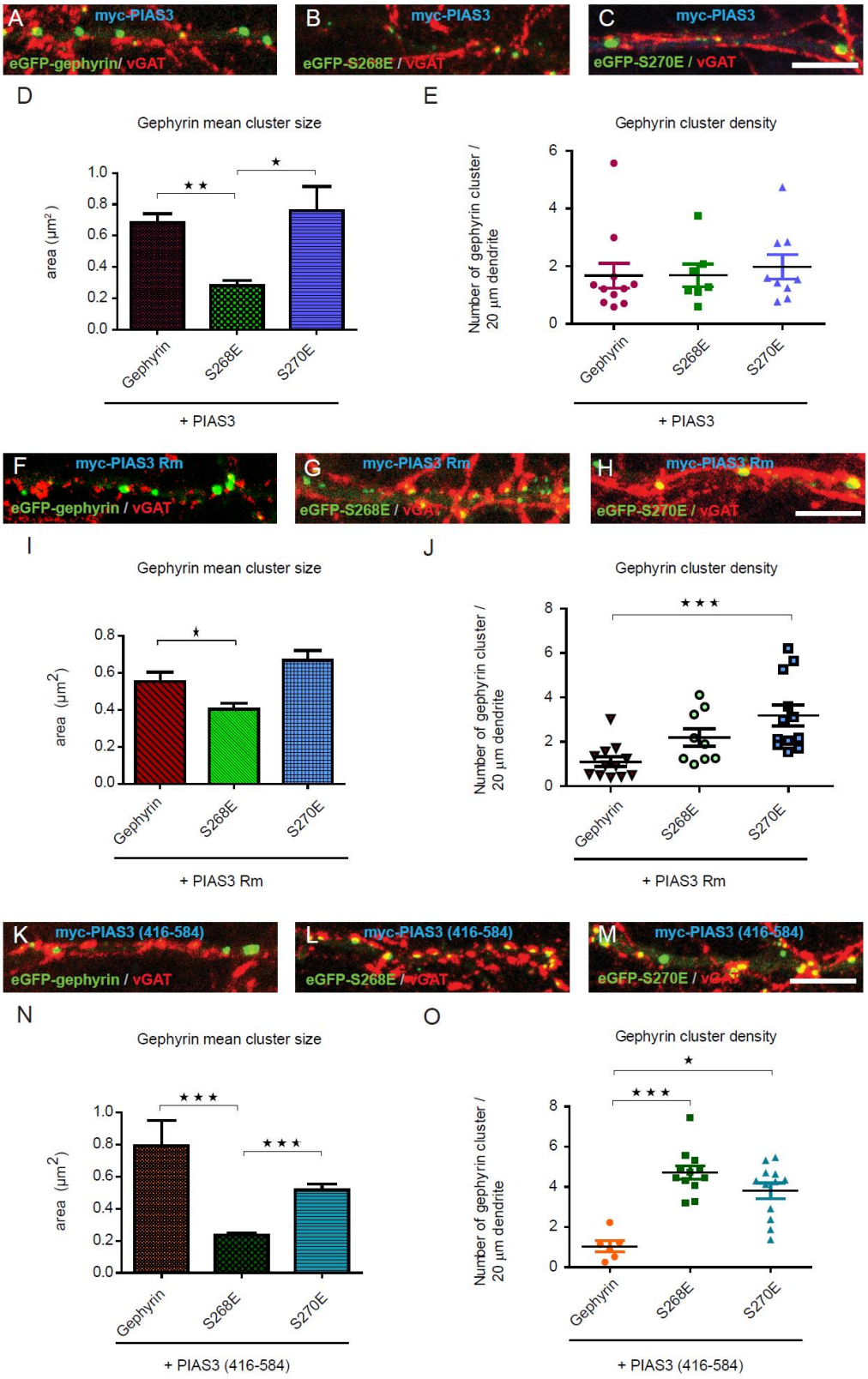
Taken together, these observations suggest that in addition to ERK1/2 modulation of PIAS-3 C-terminus, there might either be an additional GSK3β modulation site on PIAS-3. Or, the results reflect the phosphorylation status of gephyrin and the complexe cross talk existing between SUMOylation and phosphorylation PTM.

Gephyrin ERK1/2 and GSK3β site mutants are insensitive to PIAS-3Rm and C-terminus fragment

Gephyrin is an ERK1/2 substrate and it has been shown that S268 residue is phosphorylated by this kinase to modulate its scaffolding properties at GABAergic synapse [156]. Hence, we wanted to test whether inhibition of ERK1/2 pathway by PD98059 treatment in any way affected gephyrin phosphorylation site mutants. For this we co-transfected neurons with myc-PIAS-3 and eGFP-gephyrin or ERK1/2 site phospho-mimicking mutant, eGFP-268E. Morphology analysis showed eGFP-S268E is not affected by the co-expression of myc-PIAS-3 (Fig. 10D; 0.28μm²±0.034 versus 0.68μm²±0.05; KS test: $P<0.0001$). However, analysis for cluster density showed that eGFP-S268E density is reduced in the presence of myc-PIAS-3 as with eGFP-gephyrin (Fig. 10E; 1.69±0.39 versus 1.67±0.44 clusters/20μm; Mann Whitney t-test: $P=0.75$).

Figure 9: ERK1/2 and GSK3β signaling regulate PIAS-3 activity for gephyrin clustering.

(A-C) Hipocampal neurons co-transfected with eGFP-gephyrin and myc-PIAS-3 treated overnight with either ERK1/2 inhibitor PD98059 (25μM) or GSK3β inhibitor GSK3βIX (5μM) . **(D)** Quantification of mean synaptic eGFP-gephyrin cluster size, ****$P<0.0001$, KS test. **(E)** Quantifications of mean synaptic eGFP-gephyrin cluster density per 20μm dendritic length, **$P<0.01$ One-Way ANOVA. **(F-H)** Neurons co-transfected with GFP-gephyrin and myc-PIAS-3Rm treated overnight with either PD98059 (25μM) or GSK3βIX (5μM). **(I)** Quantification of mean synaptic eGFP-gephyrin cluster size, ****$P<0.0001$, KS test from the cumulative. **(J)** Quantifications of mean synaptic eGFP-gephyrin cluster density per 20μm dendritic length, **$P<0.01$ One-Way ANOVA. **(K-M)** Neurons co-transfect with GFP-gephyrin and myc-PIAS-3(416-584) treated overnight with either PD98059 (25μM) or GSK3βIX (5μM). **(N)** Quantification of mean synaptic eGFP-gephyrin cluster size. ****$P<0.0001$, KS test. **(O)** Quantifications of mean synaptic eGFP-gephyrin cluster density per 20μm dendritic length, **$P<0.01$ One-Way ANOVA. Scale bar 10μm.



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We next tested whether myc-PIAS-3Rm influenced eGFP-S268E mutant gephyrin in any way. For this, we co-transfected eGFP-S268E along with myc-PIAS-3Rm and quantified for changes in gephyrin cluster size (Fig. 10G). Quantification showed in comparison to eGFP-gephyrin, eGFP-S268E exhibited smaller cluster size in the presence of myc-PIAS-3Rm (Fig. 10I; 0.4µm²±0.03 versus 0.55µm²±0.05; KS test, *P*=0.025). eGFP-S268E cluster density was unchanged compared to eGFP-gephyrin in the presence of myc-PIAS-3Rm (Fig. 10J; 2.2±0.4 versus 1.1±0.22 clusters/20µm; Mann Whitney t-test, *P*=0.06).

We co-expressed myc-PIAS-3(416-584) C-terminal fragment along with eGFP-S268E or eGFP-gephyrin (Fig. 10K-L). Quantification showed that eGFP-S268E mutant cluster size is not influenced by myc-PIAS-3(416-584) co-expression, which is contrary to eGFP-gephyrin co-expression (Fig. 10N; 0.24µm²±0.01 versus 0.8µm²±0.16; KS test, *P*<0.0001). Quantification for cluster density also showed that eGFP-S268E is not affected by myc-PIAS-3(416-584) co-expression, in comparison to eGFP-gephyrin (Fig.10O; 4.7±0.3 versus 1.03±0.28 clusters/20µm; Mann Whitney t-test, *P*=0.0032).

These results demonstrate the importance of S268 residue in myc-PIAS-3-mediated increase in cluster size. Moreover, based on the data obtained in Fig. 9, those data suggest that ERK phosphorylation of PIAS-3C-terminus and gephyrin might be two processes occurring independently to each other.

Figure 10: Gephyrin ERK1/2 and GSK3β site mutants are insensitive to PIAS-3Rm and C-terminus fragment.

(A-C) Neurons co-transfect with myc-PIAS-3 and GFP-gephyrin **,** eGFP-S268E or GFP-S270E **. (D)** Quantification of synaptic eGFP-gephyrin cluster size, ****P<0.0001, One-Way ANOVA. **(E)** Quantification of synaptic eGFP-gephyrin cluster density per 20µm dendritic length. **(F-H) N**eurons co-transfect with myc-PIAS-3Rm and either eGFP-gephyrin, eGFP-S268E or eGFP-S270E. **(I)** Quantification of synaptic eGFP-gephyrin cluster size, *P<0.05, KS test. **(J)** Quantifications of mean synaptic eGFP-gephyrin cluster density per 20µm dendritic length. ***P<0.001 One-Way ANOVA. **(K-M)** Neurons co-transfected with myc-PIAS-3(416-584) and eGFP-gephyrin, eGFP-S268E or eGFP-S270E. **(N)** Quantification of synaptic eGFP-gephyrin cluster size, ***P<0.001, KS. **(O)** Quantifications of synaptic eGFP-gephyrin cluster density per 20µm dendritic length, ***P<0.001 One-Way ANOVA. Scale bar 10µm.

Gephyrin is phosphorylated by GSK3β at Ser270 [93]. Hence, we co-transfected myc-PIAS-3 along with eGFP-gephyrin or eGFP-S270E to observe the influence of PIAS-3 on gephyrin mutant. Quantification for eGFP-gephyrin or eGFP-S270E in the presence of myc-PIAS-3 showed increase in cluster size for both the eGFP-gephyrin and eGFP-S270E (0.68μm²±0.05 versus 0.76μm²±0.15, KS test, *P*=0.816). Quantification for cluster density showed that eGFP-gephyrin and eGFP-S270E have similar density in the presence of myc-PIAS-3 (1.67±0.44 versus 1.98±0.42 clusters/20μm; Mann Whitney, *P*=0.29).

We co-expressed myc-PIAS-3Rm along with eGFP-gephyrin or eGFP-S270E. Quantification for cluster size showed that myc-PIAS-3Rm co-expression affects eGFP-gephyrin and eGFP-S270E in a similar manner (0.55μm²±0.05 versus 0.67μm²±0.05; KS test, *P*= 0.009). However, when we quantified for cluster density change, we found that myc-PIAS-3Rm co-expression has higher density in eGFP-S270E neurons compared to eGFP-gephyrin neurons (Fig. 10H-J; 1.1±0.22 versus 3.18±0.47 clusters/20μm; Mann Whitney, *P*=0.0007). This suggests that SP-Ring domain independent mechanism of eGFP-gephyrin cluster reduction is influenced by S270 residue on gephyrin.

We also tested the effect of myc-PIAS-3(416-584) on eGFP-S270E mutant. Quantification for cluster size showed a similar phenotype to eGFP-gephyrin in the presence of myc-PIAS-3(416-584) (0.8μm²±0.16 versus 0.52μm²±0.036 KS test, *P*=0.15). When we quantified for cluster density change we find that eGFP-S270E shows an increased density compared to eGFP-gephyrin (Fig. 10M-O; 1.03±0.28 versus 3.8±0.39 clusters/20μm; Mann Whitney, *P*=0.0012). This confirms the importance of S270 site on gephyrin for PIAS-3 SP-Ring domain independent function and reduction in gephyrin cluster density. Furthermore, our data also confirm that cross-talk between SUMO and phosphorylation pathways influence PIAS-3 regulation of gephyrin.

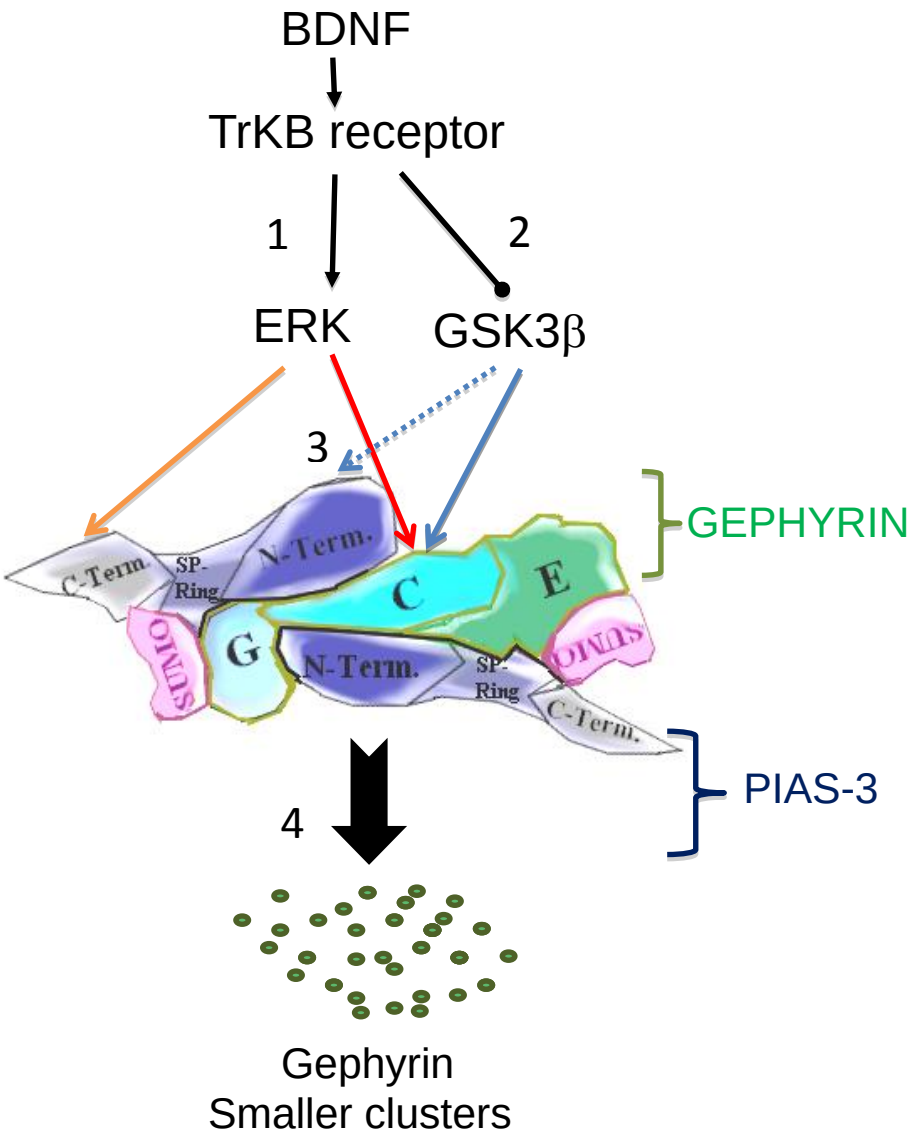

Figure 11: Schematic model of BDNF regulation of gephyrin clustering at GABAergic postsynaptic sites.

Schematic of the complex formed through the binding between gephyrin (green), PIAS-3 (blue) and SUMO (pink) and summary of the regulation of this SUMO-conjugation complex by the BDNF pathway. **(1)** BDNF via TrKB activation phosphorylates PIAS-3 (C-terminus) and gephyrin (S268) via the MAP kinase pathway, thus preventing SUMOylation to occur at K148 and/or K724 sites. **(2)** BDNF via TrKB signaling also activates PI3K/ Akt pathway, in turn inhibiting the GSK3β activity. This would promote PIAS-3 (N-terminus) de-phosphorylation, and gephyrin de-phosphorylation at S270, leading to the prevention of SUMOylation at K148 and/or K724 sites. **(3)** Inactivation of the PIAS-3 N-terminus PINIT domain, probably via GSK3β signaling, reduces PIAS-3 protein stability. **(4)** This highly dynamic system would respond to the variation in BDNF levels to regulate gephyrin scaffolds at GABAergic synapses.

Discussion

In the present study we used primary hippocampal neuronal cultures to highlight the molecular mechanism underlying acute BDNF facilitated changes at GABAergic postsynaptic sites. In this process, we uncovered a previously uncharaterized signaling pathway, SUMOylation, that converges onto the gephyrin scaffold at GABAergic synapses and is influenced by BDNF signaling . The effect of BDNF regulation of the SUMO pathway onto gephyrin scaffolding involves the SUMO E3 ligase, PIAS-3 via two independent mechanisms. One of the mechanism requires TrkB activation and the SP-Ring domain function of PIAS-3. ERK and GSK3β act downstream of TrkB to phosphorylate both PIAS-3 and gephyrin, mediating cross-talk between phosphorylation and SUMOylation pathways (Fig. 11).

Acute BDNF signaling influences kinase and SUMO pathways that converge on gephyrin scaffolds

Although in recent years several neuronal proteins have been characterized as novel SUMO-1 substrate *in vivo* [273], there is little mechanistic understanding of how SUMOylation is achieved at synaptic locations.

Previous data from our laboratory revealed a direct interaction between PIAS-3 and gephyrin for SUMO modification of gephyrin [Ghosh et al., submitted]. In the current study, our biochemical analysis identified two interaction sites for PIAS-3 on gephyrin (G and E domains). Gephyrin is SUMO-1 conjugated at the K148 and SUMO-2 conjugated at K724 residues; hence, PIAS-3 interaction close to the SUMO conjugation sites could facilitate gephyrin SUMOylation. Our data offers an elegant model for nucleo-dendritic shuttling of SUMO-1/-2, PIAS-3, SENP-2/-6 in response to BDNF signaling , thereby facilitating de-SUMOylation of synaptic proteins. BDNF-mediated translocation of these proteins is prevented byapplication of TrKB-Fc (data not shown). We also provide evidence showing chronic BDNF treatment to primary neuron culture renders proteins of the SUMO pathway insensitive to BDNF and they again enrich within the nucleus. It

is well accepted in the field that protein SUMOylation is a labile process; however, within the neuronal context our data offers a mechanistic underpinnings of a dynamic regulatory process.

Using specific SUMO-1 and SUMO-2 site gephyrin mutations (Fig. 3), and gephyrin ERK1/2 and GSK3β phosphorylation site mutations (Fig. 10), we demonstrate the convergence of these two pathways onto gephyrin scaffold upon acute BDNF signaling . In addition, the phosphorylation status at S268 and S270 residues on gephyrin renders it insensitive to PIAS-3 mediated increase in cluster size or reduction in density. This rise the question of whether SUMOylation acts upstream of phosphorylation. We have shown earlier that gephyrin phosphorylation via ERK1/2 and GSK3β pathways respectively leads to calpain-1-mediated gephyrin proteolysis and scaffold loss [258]. Taking these observations together, acute BDNF signaling -mediated downregulation of GABAergic transmission and gephyrin scaffolding can be mechanistically explained in a two step process. Step 1, BDNF activates TrkB receptors to activate ERK1/2 and GSK3β pathways that would then phosphorylate gephyrin at S268 and S270 residues, leading to calpain-1 faciliated clipping and scaffold removal from synaptic membrane. Step 2, BDNF activation of TrkB would also cause PIAS-3 and SENP-2/6 inactivation, leading to the disruption of the dynamic interplay between phosphorylation and SUMOylation pathway and preventing membrane recruitment of gephyrin.

ERK1/2 and GSK3β regulate PIAS-3 activity for gephyrin cluster regulation

In this study we present data suggesting ERK1/2 and GSK3β phosphorylation pathways affect PIAS-3 function via its C-terminus and N-terminus sequence, respectively. Inhibition of the ERK1/2 pathway provokes a significant reduction of eGFP-gephyrin clusters in the presence of PIAS-3 (Fig. 9), suggesting ERK1/2 pathway influence on PIAS-3 is essential for its action on gephyrin.

Interestingly, by looking at PIAS-3 sequence we could detect a potential ERK1/2 site in the C-terminus and a GSK3β site in the N-terminus domain of PIAS-3. Point mutation of those two siteswould confirm the data found in Fig. 9 showing the control of PIAS-3 activity by those two

kinases for regulating gephyrin cluster formation. Furthermore, ERK1/2 and GSK3β both seem to regulate differently PIAS-3 and gephyrin independently of each other. In order to help making the differences, it would be proposed to test the co-transfection of primary hippocampal neurons with myc-PIAS-3 full length and either eGFP-S268A with or without PD98059 treatment or eGFP-S270A with or without GSK3βIX treatment. Untreated transfected cells would provide some evidence on the fact that ERK1/2 and GSK3β act directly on PIAS-3 when gephryin is dephophorylated by either of them. In addition, repeating the proposed experiment by replacing myc-PIAS-3 with myc-PIAS-3Rm or myc-PIAS-3(416-584) might confirm our speculation consisting of ERK1/2 affecting PIAS-3C-terminus and GSK3β acting on PIAS-3 N-terminus.
Moreover, if we take in consideration that ERK1/2 phosphorylates PIAS-3 on its C-terminal leading to a normal function of PIAS-3 therefore increasing gephyrin cluster size, phophorylation of gephyrin at S268 site might not occur at the same time. Furthermore, as BDNF signaling leads to activation of ERK1/2 and down-regulation of gephyrin cluster size, it is therefore possible that activated ERK1/2, under BDNF signalling, would only act on gephyrin.

PIAS-3 is a specific inhibitor of transcription factor STAT-3, and diverse signaling pathways like IL6, EGF, NGF etc. activate STAT-3-induced gene transcription [216, 274]. The phosphorylation of STAT-3 at Y705 and S727 via JAK and ERK1/2, respectively, are well documented. STAT-3 phosphorylation at Y705 leads to PIAS-3/ STAT-3 complex formation and nuclear translocation, causing a reduction of STAT-3 mediated gene expression [275]. In a model of myocardial infraction for sympathetic nerve sprouting, it was shown that NGF phosphorylates STAT-3 at S727 via ERK1/2, promoting nerve regeneration in a STAT-3 transcription-independent fashion. Furthermore, in the same study STAT-3 (pS727) was found to be localized both in the nucleus and neuronal processes [274]. It is likely that BDNF activation of ERK1/2 causes STAT-3 (S727) phosphorylation leading to PIAS-3 binding and sequestration of PIAS-3 activity. Taken together our data point towards a non-transcriptional role for PIAS-3 in gephyrin scaffold regulation.

It is conceivable that ERK and GSK3β pathways directly phosphorylate PIAS-3 affecting its conformation and/or stability. In support of this idea, we provide data showing that both full length PIAS-3 and PIAS-3 C-terminal sequence (AA 416-584) are influenced by the inhibition of the ERK1/2 pathway (Fig. 9). Furthermore, introduction of point mutations into the N-terminal PINIT domain (Fig. 8) or inhibition of GSK3β (Fig. 9) is sufficient to reverse PIAS-3-mediated gephyrin cluster regulation. The C-terminus sequence and the N-terminal PINIT domain within PIAS-3 could influence the SP-Ring domain function via 3D protein confirmational change, thereby activating the SUMO-independent pathway, via currently unknown mechanisms.

BDNF signaling and gephyrin modulation for brain network integrity

Dynamic regulation of gephyrin scaffolding at GABAergic synapses is especially relevant within the context of synaptic homeostasis, wherein individual neurons and/or synapses adapt to alterations in afferent activity. It has become increasingly clear that GABAergic inhibition is a dynamic process with rapid changes in gephyrin scaffolding in response to incoming stimuli; however, the underlying mechanisms for such adaptations *in vivo* are unknown. Our study not only characterizes the signaling network influencing specific PTM(s) on gephyrin, but, by identifying specific residues on gepyhrin at single amino acid resolution, we offer functional insights at unprecedented depth. It would be a major aim to investigate wether this mechanism still operates under pathological conditions, such as in ischemia, where elevated BDNF levels are known to downregulate GABAergic transmission, leading to adaptative changes and cognitive impairement [104].

In the context of ischemia, an elevation of the general level of SUMO-conjugated proteins as well as a significant increase of BDNF transcript and protein levels have been reported after an ischemic stroke [220, 242, 276-278]. However, these studies failed to explore possible link between BDNF and SUMO pathway. Our work provides the first evidence of BDNF regulates the SUMO pathway. Interestingly, the hippocampal formation is more sensitve to ischemic stroke

in comparison to other brain areas [279-281]; and one sees elevation in BDNF levels within hippocampal formation after stroke. Thus, it is conceivable that under ischemic conditions BDNF signals to promote gephyrin SUMOylation to dampen GABAergic inhibition post-ischemic injury.

Chapter 2: SUMOylation-defective gephyrin mutant stabilizes GABAergic synapses in post-ischemic CA1 hippocampal neurons

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ZST performed experiments, data analysis and wrote the manuscript. RG performed experiments and data analysis. P.K-YC performed some experiments and data analysis. R.A.M helped with project design and data analysis. SKT designed the project and helped writing the manuscript.

Abstract

We have previously demonstrated that BDNF facilitates structural adaptations of gephyrin clusters at GABAergic postsynaptic sites trough activation of TrKB and modulation of gephyrin post-translational modification. Besides its key role during brain development, BDNF was shown to play an important role in recovery after an ischemic injury. The goal of the present study was to test whether BDNF mediates its effects by regulating GABAergic transmission and gephyrin clustering, using OGD as a model of ischemia in hippocampal organotypic slice cultures.

We report that a brief period of OGD in mature slices induces both morphological and functional changes at hippocampal CA1 pyramidal neurons. OGD decreases surface expression of gephyrin and $GABA_A$Rs-containing $\alpha 1$ and $\alpha 2$ subunits. Subsequently, OGD decreases GABAergic mIPSCs frequency. Interestingly these morphological and functional deficits recover 1 week post-OGD. Moreover, we provided evidence that these deficits were consequences of BDNF signaling activation as we could prevent them by the use of the BDNF scavenger TrKB/Fc. Furthermore, we could demonstrate that the availability of gephyrin expression was dependent of its SUMOylation regulation.

Altogether, these results revealed the importance of BDNF regulation of gephyrin post-translational modification to induce GABAergic synaptic plasticity, likely contributing in the recovery phase post-ischemic injury.

Introduction

Homeostatic synaptic plasticity is a fundamental mechanism through which neurons adjust the strength of their synapses in response to global changes in neurotransmission. In order to maintain synaptic homeostasis, both excitatory and inhibitory circuits undergo adaptive changes affecting pre- and postsynaptic function. Changes in neuronal activity regulate a complex network of signaling cascades, which in turn adjust synaptic function. These signaling cascades induce post-translational modifications of synaptic proteins to regulate their targeting localization and functions. One of the major synaptic proteins undergoing PTM and in turn modulating synaptic function is the scaffolding protein. Scaffolding proteins can target, localize and build complexes of functionally related proteins to facilitate the formation of presynaptic active zone and postsynaptic density. The main scaffolding protein at GABAergic (and glycinergic) synapses is gephyrin, a highly conserved multifunctional protein that has been hypothesized to be the facilitator of postsynaptic density [18].

Molecular mechanisms underlying gephyrin inter- and intra-molecular interactions are slowly emerging. In recent years multiple phosphorylation residues have been identified on gephyrin [93, 151, 156, 157]. However, understanding these PTMs has allowed us to explore the molecular mechanisms underlying gephyrin regulation and to determine how this regulation impacts the formation and plasticity of GABAergic synapses. It was recently reported that diverse PTMs on gephyrin such as phosphorylation, SUMOylation and acetylation regulate gephyrin scaffolding at GABAergic synapse. In the same study it was also shown that ERK1/2 and GSK3β kinase pathways cross-talk with the SUMO pathway to modulate gephyrin scaffolding [Ghosh et al., submitted].

The heterologous SUMO-1/-2/-3 proteins are conjugated to cellular substrates in three steps: step one: SUMO protein is activated by the E1 activating enzyme Sae2/Uba2; step two: SUMO is transferred to the E2 conjugating enzyme Ubc9; step three: SUMO is ligated onto the substrates via E3 ligase PIAS family of proteins. SUMO conjugation is reversible and is facilitated via the function of sentrin family of SENP proteins. Although the SUMO pathway was discovered as a key regulator of DNA damage response [282], in the last decade SUMOylation of synaptic proteins has emerged as a critical regulator of neuronal plasticity [176, 220, 221]. It has been

demonstrated that protein SUMOylation increases with cellular stress [240]. Hence, several independent studies have demonstrated a transient increase in the overall levels of SUMO-conjugated proteins in the brain following injury such as stroke [253, 283].

During stroke, a brief period of ischemia causes cell death in the infarct area, causing disruption of network homeostasis via alterations at both excitatory and inhibitory synapses [284-286]. While mechanism(s) underlying synaptic changes at glutamatergic terminals is well characterized in literature [287-289], we lack a mechanistic understanding for changes at GABAergic synapses post ischemia [252, 290, 291]. In hippocampal formation $GABA_A$Rs are down-regulated post ischemia, further exacerbating the excitotoxicity related cell death [248]. The observed downregulation of $GABA_A$Rs from inhibitory synaptic terminals is linked to the regulation of gephyrin scaffolding via a currently unknown mechanism [252].

In the current study we demonstrate that ischemia increases *bdnf* transcription specifically in CA1 area of the hippocampus, leading to GABAergic synapse loss via gephyrin regulation. We use molecular biology, confocal microscopy and electrophysiology to investigate the mechanistic basis for gephyrin regulation under ischemic conditions. Our data identifies BDNF-induced GABAergic synaptic plasticity via gephyrin post-translational modification. Using specific gephyrin point mutations that are defective for SUMO-1 or SUMO-2 conjugation we demonstrate the importance of this PTM for ischemia-induced plasticity at GABAergic synapses.

Material and Methods

Organotypic hippocampal slice cultures

All experiments were performed in accordance with guidelines from the Swiss Vetinerary office, Canadian Council on Animal Care and the National Institutes of Health in the USA. They were all approved by the Animal Resource Committee of the School of Medicine at McGill University. Organotypic hippocampal slices (400μm thickness) were obtained from post-natal day 7 C57BL/6J mice or transgenic mice expressing MARCKs-enhanced GFP tagged to the CA1 neuronal membrane. Tissue slices of 400μm thickness were prepared following the roller-tube method from Gähwiler technique [292, 293]. The slices were incubated in an antibiotic-free serum medium containing 25% heat-inactivated horse serum, 25% Hank's balanced salt solution, and 50% Basal Medium Eagle. They were maintained during 3 weeks minimum allowing maturation prior to experimentation at 36°C in a roller drum incubator.

Organotypic slice transfection

eGFP-gephyrin, eGFP-K148R or eGFP-K724R and pCR3-Td-Tomato were co-transfected into 14 days in vitro (DIV 14) organotypic slices using the Helios Gene Gun (Bio-Rad laboratories), following the vendor protocol.

Oxygen Glucose Depravation (OGD) treatment

The slices were incubated in glucose-free Tyrode (ACSF) solution supplemented with 2mM 2-deoxyglucose (2-DG), 8mM sucrose, and 3mM sodium azide (NaN_3) and bubbled with 95%N_2 /5%CO_2. The slices were incubated during 4min in the OGD solution or normal Tyrode solution (control conditions) and returned in normal culture medium 90min, 24H or 1week before experimenting as a model for ischemic injury *in vitro* [294].

Pharmacological treatments: BDNF scavenger: TrKB/Fc Chimera (1μg/mL, R&D Systems #688-TK-100).

Immunohistochemistry

Slices were fixed with 4% paraformaldehydes (PFA) during 1 hr and washed with 0.1M phosphate buffer, prior to permeabilization with 0.4% Triton x100 and subsequently blocked with 1.5% heat-inactivated horse serum overnight at 4°C. The primary antibody incubation (in permeabilizing buffer) was performed over 5 days at 4°C. The slices were then washed several times with 0.1M PBS during the whole day, followed by the incubation with the secondary antibody mixture overnight at 4°C. Slices were mounted using Dako Fluorescence Mounting medium (Dako Canada).

Antibodies

Mouse anti-Gephyrin (1:500, clones mAb7a, Synaptic Systems #147021), rabbit anti-SUMO-1 (1:250, Abcam#ab11672), rabbit anti-SUMO-2/3 (1:250, Abcam#ab109005), mouse anti-PIAS-3 (1:500, Sigma #P0117), rabbit anti-NeuN (Millipore #MAB377X). All the secondary antibodies were from Jackson ImmunoResearch: Goat anti-Mouse Cy3 IgG (1:250, #115165), Goat anti-Mouse IgG Cy5 (1:250, #115175), Goat anti-Rabbit IgG Cy3 (1:250, #111165) and Goat anti-Rabbit IgG Cy5 (1:250, #111175).

Microscopy and Image processing

Images were acquired on a Leica DM6000B laser scanning microscope (Leica Microsystems) with objective lens 63x NA 1.4 oil immersion. A minimum of 3 slices from 3 independent batches per condition were acquired using a z-stack (0.3µm step size). Image analyses of gephyrin clustering in the hippocampal CA1 region were done, post-deconvolution with Hygens Essential software, using the Surpass and the Spot functions of Imaris 7.00 software (Biplane AG).

Images for PIAS-3 quantifications were acquired on confocal laser scanning microscope (LSM 710, Carl Zeiss) with objective lens of 40x or 63x with a pinhole set at 1 Airy unit and a pixel size of 0.13µm. A minimum of 9 cells from 3 independent batches per condition were acquired

using a z-stack (0.5µm step size). Image analyses were performed with a custom written analysis for Image J software using maximal intensity z-projected images.

Electrophysiological recordings

Cells were held at a potential of -60mV with an Axopatch 200A amplifier (Molecular Devices) and perfused with a Tyrode solution containing: 137mM NaCl; 0.4 mM $NaH_2PO_4.H_2O$; 2.7 mM KCl; 2.5 mM $CaCl_2.H_2O$; 2 mM $MgCl_2$. H_2O; 11.6 mM $NaHCO_3$; 5.6 mM D-glucose (pH7.4, bubbled with 95%O2 and 5%CO2)**.** Spontaneous GABAergic miniature postsynaptic currents (mIPSCs), from CA1 pyramidal neurons, were isolated by batch application of Tetrodotoxin (TTX 1µM, Tocris); 3-[(R)-2-carboxypiperazin-4-yl]-propyl-1-phosphonic acid (CPP 25µM, Tocris); 6-cyano-7-nitroquinoxaline-2.3-dione (CNQX 5µM, Tocris); CGP55845 hydrochloride (5µM, Tocris); Strychnine (0.3µM, Tocris).

Whole-cell voltage-clamp recordings were performed at room temperature using borosilicate glass microelectrodes (3-5MΩ, GC150TC, Clark Instruments) refilled with an internal solution containing: 140 mM CsCl, 4 mM NaCl, 5 mM EGTA, 10 mM HEPES, 0.5 mM $CaCl_2$, 0.5 mM Na-ATP, 2 mM Mg-GTP, 2 mM QX-314 (pH adjusted to 7.4 with CsOH). The cells were monitored for a min of 5min, after whole-cell configuration, for equilibration of the internal solution and ensure that the seal and opening were maintained. The cells were then recorded during 5 stable minutes.

mIPSCs data acquisition was performed using pClamp 10 (Molecular devices) and recorded with a filtering of 2KHz and digitized at 20KHz with the Axon Digidata 1440 digitizer (Molecular devices). From every experiment, 3min of synaptic events from a minimum of 8 cells were analysed using Mini Analyses software (Synaptosoft).

Real Time qPCR

Areas CA1 and CA3 were micro-dissected from 5 to 6 slices from 3 independent litter and used for each experimental conditions. Total mRNA was extracted using BioRad extraction kit. Subsequently, 1µg of mRNA was reverse transcribed to cDNA following the manufacturer's

protocol (Roche Diagnostic). The RT-qPCR was performed using 30ng of cDNA in a 20µL reaction mixture containing EVA green mastermix (Solis BioDyne #08-24-00008). All qPCR reactions were performed under those conditions: 40 cycles; denaturation at 95°C for 15s, annealing at 62°C for 25s and extension at 72°C Primers. The following primer pairs were used for each reaction: bdnf Fwd: 5'-TGC AGG GGC ATA GAC AAA AGG-3', Rev: 5'-CTT ATG AAT CGC CAG CCA ATT CTC-3'; Sumo-1 Fwd: 5'-GGCAAAACCTTCAACTGAGGA-3', Rev: 5'-CTCCATTCCCAGTTCTTTCGG-3'; Sumo-2 Fwd: 5'-ATTTGAAGGTGGCGGGAC-3', Rev: 5'-CTGTTTCGTTGATTGGCTGC-3'; *PIAS-3* Fwd: 5'-GGA TGG TCT CCA GTA CAG CG-3', Rev: 5'-CAG GAA GTG GGA AGG GGT TC-3' and *Gapdh* Fwd: 5'-TGCCCCCATGTTTGTGATG-3' Rev: 5'-TGTGGTCATCAGCCCTTCC-3'.

Statistical analyses

Numerical data are expressed as mean ± standard error of the mean (SEM). Statistical analyses of mean values were made, when appropriate, using the Student's T-test of GraphPad Prism software. $P<0.05$ was considered statistically significant.

Except for PIAS-3 puncta size in addition to cumulative probability distribution of mIPSCs (amplitude and IEI) were analysed by pair-wise using a Kolmogorov-Smirnov test. $P<0.05$ was considered statistically significant.

Results

Organotypic cultures preserve the hippocampal network architecture, allowing the study of activity-dependent changes in a physiologically relevant context and also serve as a model for distinct pathological states [295]. In particular, OGD is considered to be a good *in vitro* model to mimic cerebral ischemia and study hippocampal synaptic plasticity at different time-points after the injury. Hence, we used organotypic hippocampal slice cultures and OGD to study the molecular basis of post-ischemic GABAergic synapse plasticity in the CA1 area.

OGD-induced loss of gephyrin clusters recovers after one week

As proof of principal, we tested our organotypic slice culture model for hippocampal OGD, by staining for propidium iodide (PI), a marker for cell death and for hypoxia-inducible factor 1α (HIF1α) [279-281, 296].

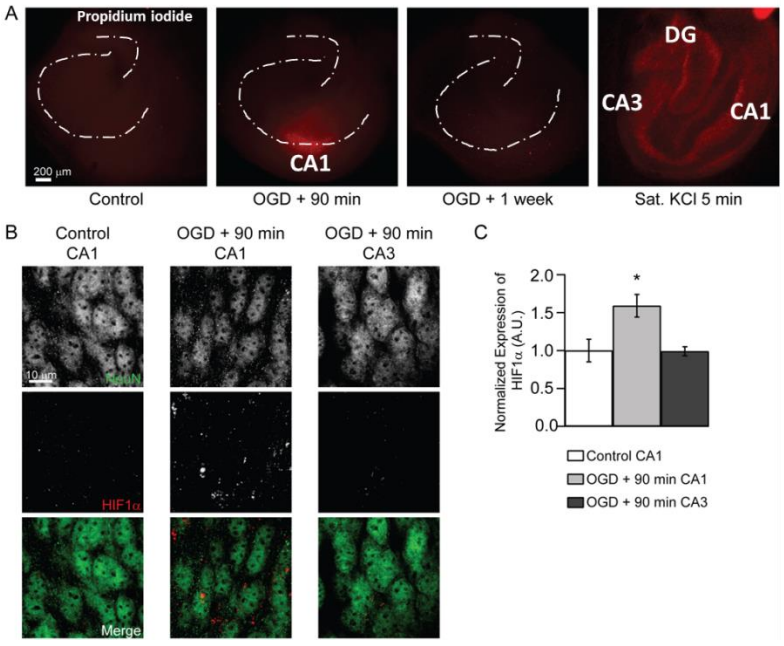


Figure 1: Example of OGD inducing neuronal cell death accompanied by an increase in HIF1α.

(A) Staining for cell death using propidium iodide (red) in control+ 90min, OGD+ 90min, OGD+ one-week in organotypic hippocampal slice cultures. Control experiment using saturated KCL concentration. Scale bar, 200µm. **(B)** Staining for HIF1α (white) in CA1 and CA3 in control+ 90min and OGD+90min. **(C)** Normalized mean intensity fluorescence of HIF1α .*$P<0.05$ Student t-test. Scale bar, 10µm.

We found that almost all the cultures showed elevated staining for HIF1α, however, only 20% of are immune-positive for PI. These observations confirm that in our model we can successfully induce ischemia with little cell death (Fig. 1A, B).

Since we are interested in understanding both short- and long-term adaptations at GABAergic synapses after transient OGD, we exposed the slices to 4 min OGD, followed by either 90 min or one-week recovery prior to staining for gephyrin clusters. At 90 min post-OGD, we observed an overall decrease in the density of gephyrin clusters (Fig. 2C; OGD+90min 40.08±8.22 clusters/ 512x512μm image) compared to control+90min (Fig. 2A, F; 494.9±29.33, Two-tailed Mann Whitney-test $P<0.0001$). This difference represented a disappearance of 90% of gephyrin clusters from CA1 neurons. We also analyzed for changes in gephyrin cluster volume, as our earlier studies have shown this to be a direct correlate for availability of synaptic $GABA_A$Rs [93, 156]. The decrease in cluster density post-OGD is accompanied by a decrease in the average cluster volume of the remaining gephyrin clusters at OGD+90min ($0.09\mu m^3\pm0.01$) compared to control+90min (Fig. 2E; $0.27\mu m^3\pm0.04$, Two tailed Mann Whitney-test $P=0.0008$). Interestingly, all the remaining gephyrin clusters where apposed to vGAT (data not shown), which might also suggest that the synapses are likely functional and the presynaptic interneurons might not be spared (or not totally). We could also notice that many of the remaining gephyrin clusters have a large cluster volume.

Importantly, the gephyrin clusters density recover one-week after OGD (Fig. 2D; OGD+1week 413.67±33.7) compared to control+ one-week (Fig. 2B, F; 475.14±29.16, Two-tailed Mann Whitney-test $P=0.27$), suggesting the existence of a mechanism for re-establishing gephyrin clusters at GABAergic PSD during the recovery process. Moreover, gephyrin cluster volume also showed recovery after one-week recovery (OGD+1week $0.21\mu m^3\pm0.03$) compared to control+1week (Fig. 2B, F; $0.17\mu m^3\pm0.01$; Two-tailed Mann Whitney-test $P=0.72$). Interestingly, our control cultures did not show any significant differences in gephyrin cluster density (Fig. 2F; Two-tailed Mann Whitney-test $P=0.82$) or cluster volume (Fig. 2E; Two-tailed Mann Whitney-test, $P=0.074$).

Based on these initial observations, we could ascertain that in our OGD model gephyrin immunopositive expression dramatically disappeared and reappeared probably contributing to plasticity changes at GABAergic synapse.

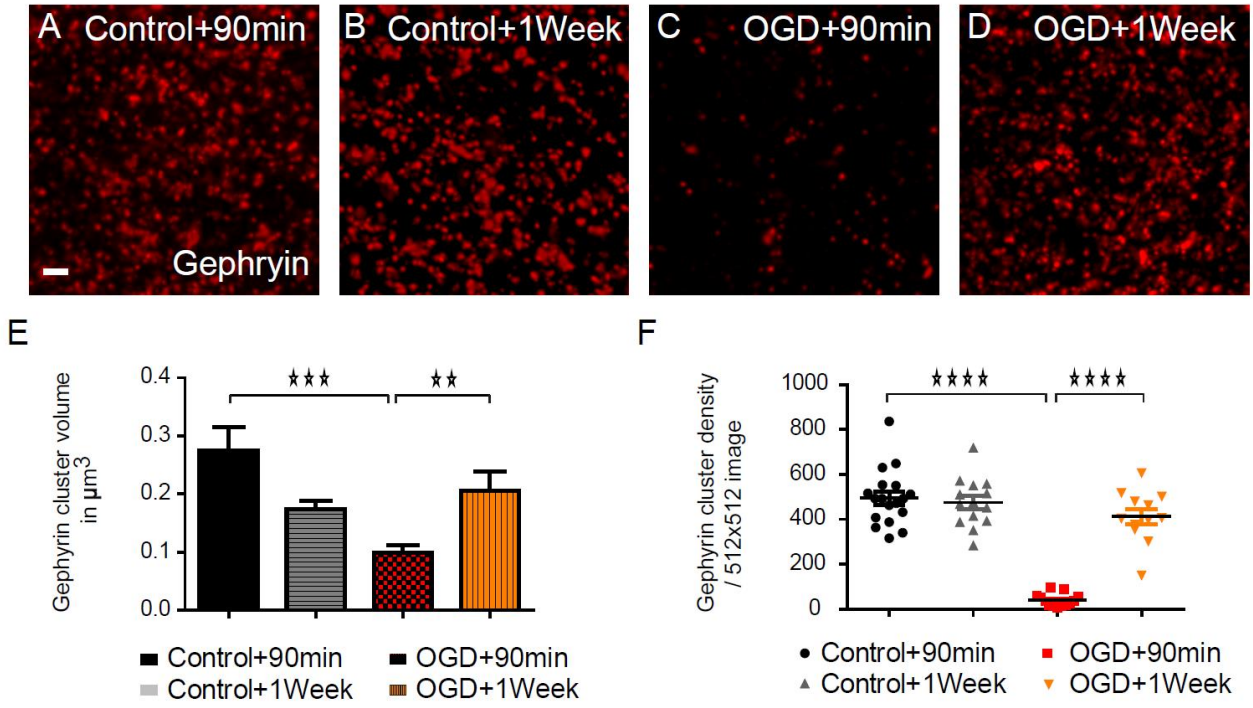


Figure 2: Gephyrin cluster loss post-OGD recovers after 1Week.

(A-B) Immunofluorescence staining for endogenous gephyrin in mock-treated DIV 21 and DIV 28 organotypic hippocampal slice cultures. **(C-D)** Immunofluorescence staining for endogenous gephyrin after 90 min and one-week recovery post-OGD. Scale bar =2µm. **(E)** Gephyrin cluster volume in μm^3 per confocal stack (512x512 pixel images), ***P<0.001 One-Way ANOVA followed by a Kruskall-Wallis test. **(F)** Gephyrin cluster density per confocal stack, ****P<0.0001 One-Way ANOVA followed by a Kruskall-Wallis test.

Blocking BDNF signaling prevents gephyrin clusters after OGD

Many independent studies have demonstrated BDNF upregulation post-OGD [276, 297]. Hence, we wanted to determine whether BDNF signaling induces gephyrin clustering loss after OGD. TrkB is the high affinity receptor for BDNF; we used TrKB/Fc, a chimera made of the extracellular domain of TrKB receptor and an Fc fragment of human IgG to scavenge exogenous BDNF.

TrKB/Fc (1µg/mL) was added immediately after transient OGD in the culture medium and the slices were placed in the incubator for 90min before proceeding with morphology analysis. The addition of TrKB/Fc to control slices (Control+TrKB/Fc) did not influence gephyrin cluster density (Fig. 3A, D; 311.9±25.6 versus 331.9±22.37, Two-Tailed Mann Whitney T-test *P*=0.62) and cluster volume (Fig. 3A, C, 0.16µm^3±0.008 versus 0.16µm^3±0.008, Two-Tailed Mann Whitney T-test *P*=1.0). OGD cultures replicated the significant decrease in the gephyrin cluster density compared to untreated control (Fig. 3D; 47.44±8.78 versus 331.9±22.37, Two-Tailed Mann Whitney T-test *P* <0.0001). In addition, OGD samples show a significant reduction of gephyrin cluster volume compared to control+90min, (Fig. 3C; 0.098µm^3±0.017 versus 0.16µm^3±0.008, Two-Tailed Mann Whitney T-test *P=0.0062*).

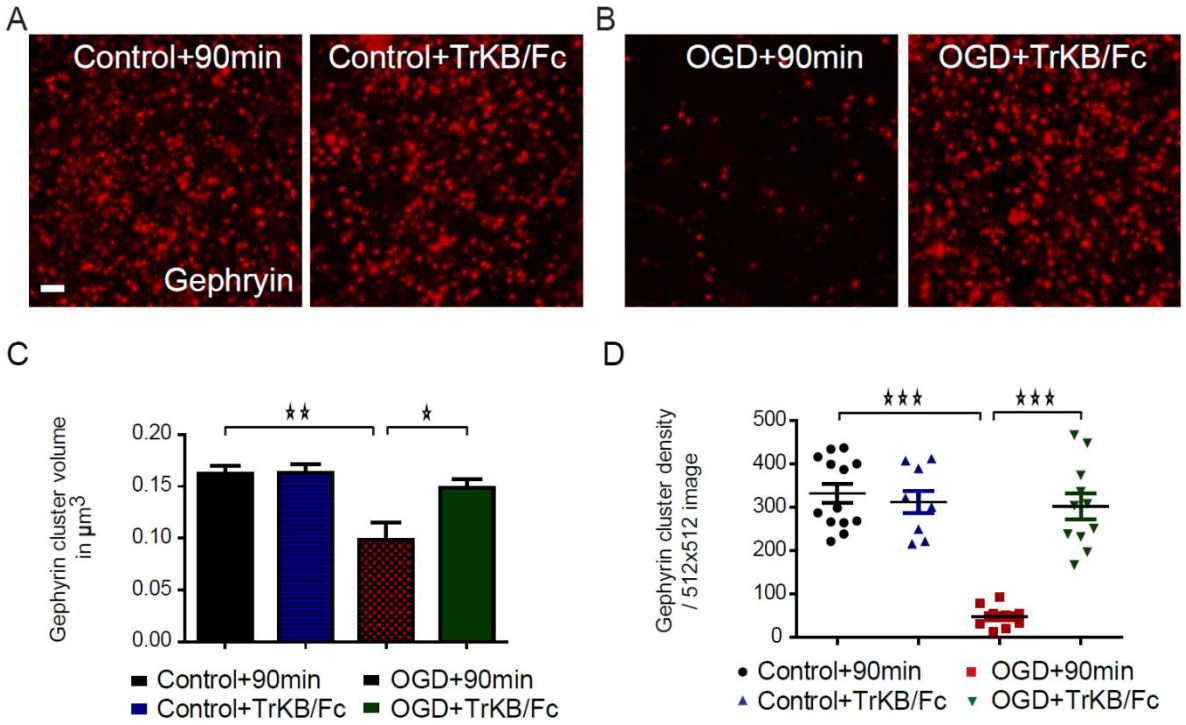


Figure 3: Blocking BDNF signaling post-OGD rescues gephyrin clustering in neurons.

(A) DIV 21 organotypic hippocampal slice showing endogenous gephyrin clusters in control untreated neurons and in neurons treated with 1µg/mL of TrKB/Fc post-90min treatment. Scale bar 2µm. **(B)** DIV 21 organotypic hippocampal slice showing endogenous gephyrin clusters 90min post-OGD and in neurons treated with TrKB/Fc for 90min post-OGD. **(C)** Gephyrin cluster volume in µm^3 per confocal stack (512x512 images), **P*<0.05 One-Way ANOVA followed by a Kruskall-Wallis test. **(D)** Gephyrin cluster density per confocal stack, *****P*<0.0001 One-Way ANOVA followed by a Kruskall-Wallis test.

Samples treated with TrKB/Fc showed a prevention of gephyrin cluster density (Fig. 3B, D; 302±29.5 versus 47.44±8.78, Two-Tailed Mann Whitney T-test *P*=0.0002) and volume (Fig. 3C; 0.15µm^3±0.008 versus 0.098µm^3±0.017, Two-Tailed Mann Whitney T-test *P*=0.036). These observations confirm a role for BDNF in OGD-induced gephyrin cluster reduction in hippocampal CA1.

The OGD treated slices are very fragile after 90 min recovery; to increase data reproducibility we tested organotypic cultures for gephyrin cluster loss 24 hr post-OGD (Fig. 4A). Similar to the 90 min samples, 24 hr recovery also showed a significant loss of endogenous gephyrin clusters density (Fig. 4B; 59.78±7.3 versus 210.9±15.7, Two-Tailed Mann Whitney T-test *P*<0.0001) suggesting that many of the impairment observed at 90 min persists at 24 hr post-OGD. However, the remaining gephyrin clusters show basal cluster volume (Fig. 4C; 0.096µm^3±0.008 versus 0.091µm^3±0.006; Two-Tailed Mann Whitney T-test *P=0.63*).

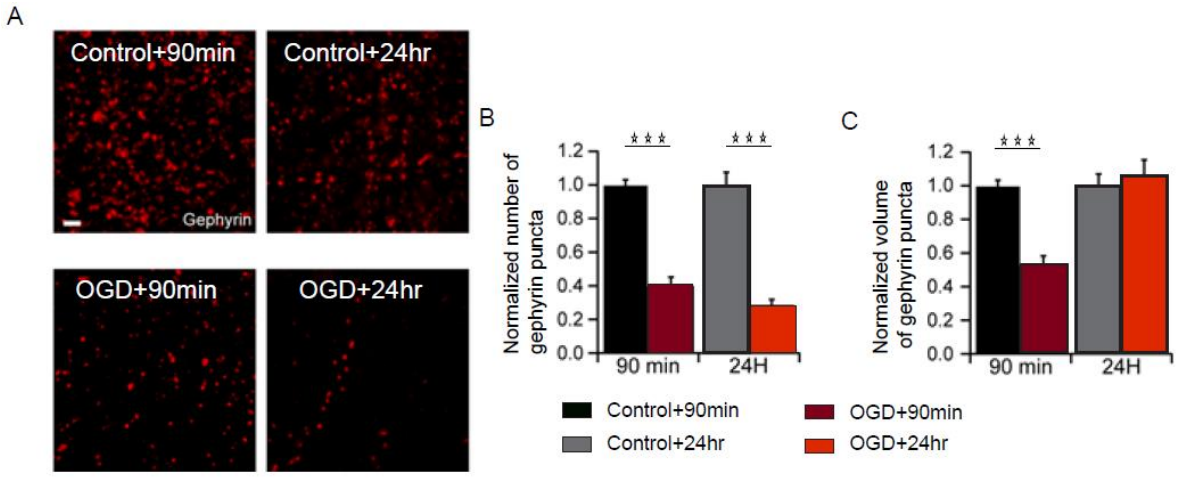


Figure 4: Following gephyrin cluster loss post-OGD after 24hr recovery.

(A) Immunostaining for endogenous gephyrin at 90min and 24hr post-OGD. Scale bar 2µm. **(B)** For better visualisation of the differences the results has been normalised. Normalized gephyrin cluster density per confocal stack, ***P<0.0001 Paired Student t-test. **(C)** Normalized gephyrin cluster volume in µm^3 per confocal stack (512x512 images), ***P<0.001 Paired Student t-test.

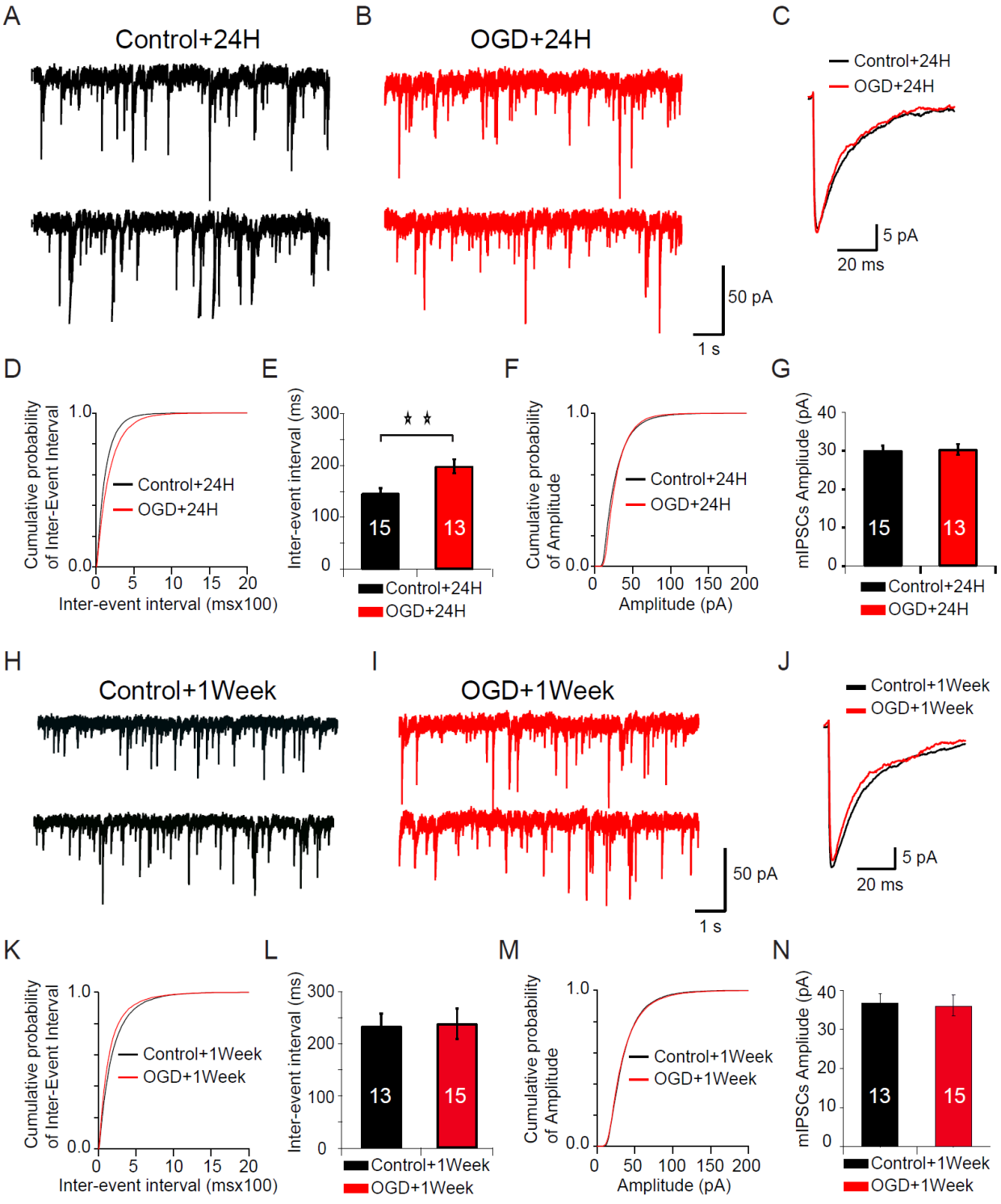
Functional reduction of GABAergic transmission after OGD

We have consistently shown in the past that morphological alterations in gephyrin scaffolding can be a direct correlate for functional changes in GABAergic transmission [93, 156]. Hence, we wondered whether OGD-induced impairment in gephyrin clustering also resulted in reduced GABAergic transmission. For this we performed whole-cell patch-clamp recordings of miniature inhibitory postsynaptic currents (mIPSCs) of CA1 pyramidal neurons 24 hr after OGD.

Analysis of GABAergic mIPSCs showed a significant increase in the mean inter-event interval (IEI) 24 hr post-OGD compared to controls (Fig. 5A-E; 198.2ms±13.34, n=13cells versus 146.0ms±10.25, n=15, Two-tailed Unpaired T-test P=0.0046). Cumulative probability distribution confirmed the reduction in IEI with a rightward shift in curve (Fig. 5D; KS test P<0.01). When we compared for differences in mIPSCs amplitude we found that 24 hr post-OGD the amplitude of the remaining mIPSCs were unaffected (Fig. 5G; 30.34pA±1.403, n=13 versus 30.05pA±1.231, n=15, Two-tailed Unpaired T-test P=0.88). Cumulative probability distribution curves for the amplitude were also similar (Fig. 5F). Our data suggest that the OGD-induced reduction of mIPSC frequency (35%) correlates with the decrease in gephyrin clusters in CA1 neurons. Consistent with these observations, staining for α1 (Fig. 6A, C; Paired Student's T-test P=0.019) or α2 $GABA_A$Rs (Fig. 6B, D; Paired Student's T-test P<0.001) also shows a similar reduction of 30% from CA1 pyramidal neurons.

Figure 5: Defects in GABAergic mIPSCs correlate with morphological loss of gephyrin clusters.

(A-B) Example traces of GABAergic mIPSCs recording form CA1 hippocampal neurons control neurons (DIV 21, n=15 cells) and neurons after 24 hr recovery post-OGD (OGD+24h, n=13). **(C)** Average traces of mIPSCs (Two-tailed Unpaired T-test). **(D)** Cumulative probability distribution of mIPSCs inter-event interval (IEI), ***P<0.01* Kolmogorov-Smirnov (KS) test. **(E)** Mean mIPSCs IEI. **(F)** Cumulative probability distribution of amplitudes in control and neurons after 24 hr recovery post-OGD. **(G)** Quantification of mean mIPSCs amplitudes (Two-tailed Unpaired T-test). **(H-I)** Example traces of mIPSCs recording from control neurons (DIV 28, n=13) and neurons one-week post-OGD (n=15). **(J)** Average traces of mIPSCs. **(K)** Cumulative probability distribution of mIPSCs IEI in control neurons and neurons one-week post-OGD. **(L)** Quantification of mean mIPSCs IEI (Two-tailed Unpaired T-test). **(M)** Cumulative probability distribution of mIPSCs amplitude. **(N)** Quantification of mean mIPSCs amplitudes.



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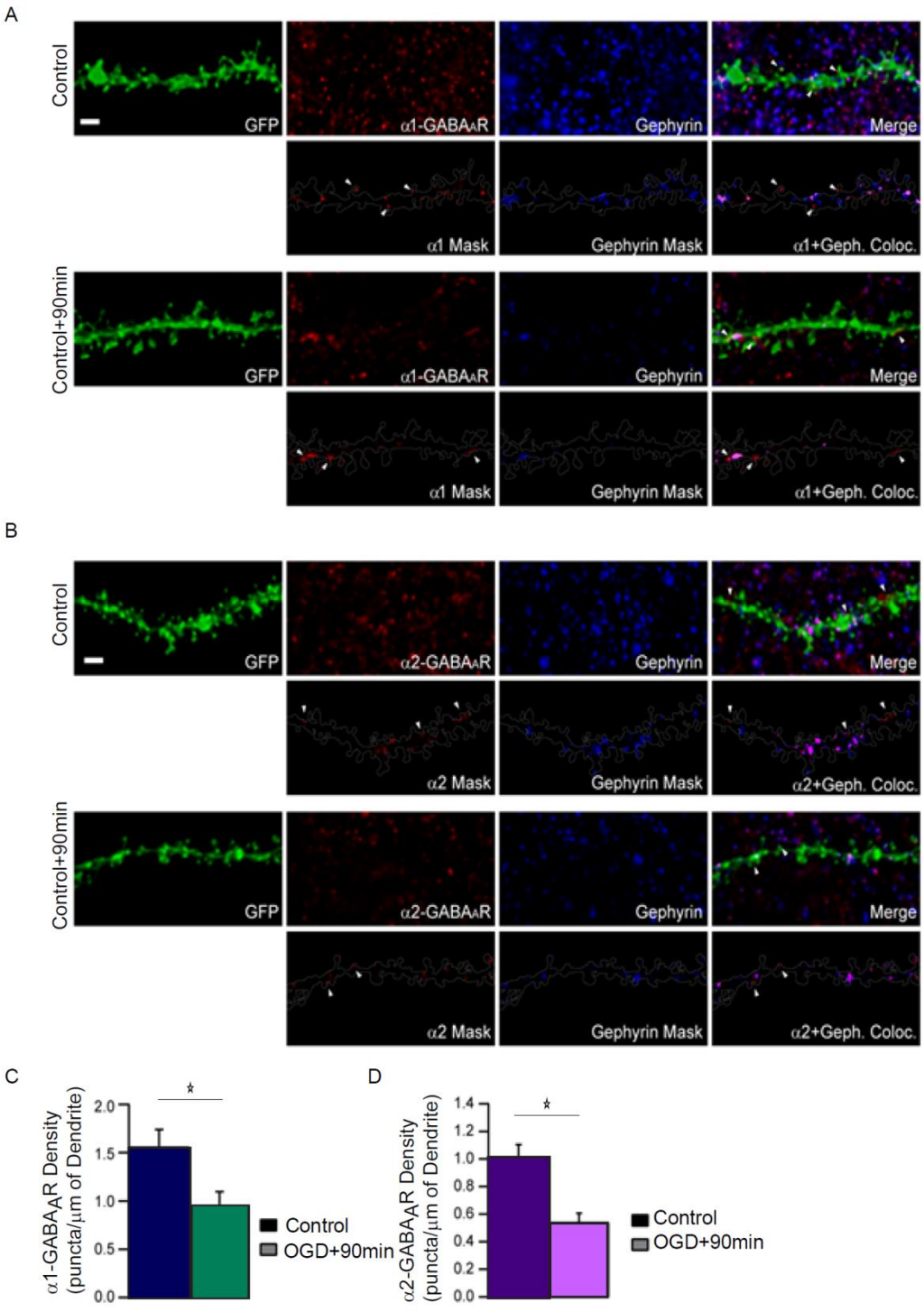


Figure 6: OGD reduces α1- and α2- containing $GABA_ARs$.

(A-B) DIV 21 organotypic hippocampal slice stained for endogenous gephyrin (blue) and endogenous α1-or α2-containing $GABA_ARs$ subunits (red) in untreated and OGD treated neurons. Scale bar 2 μm. **(C, D)** Quantifications of mean puncta density of α1- and α2- containing $GABA_ARs$ per μm dendrite. **P*<0.01 Paired t-test.

Our morphological analysis one-week post-OGD showed that gephyrin cluster density returns to baseline levels. Hence, we wanted to test whether GABAergic mIPSCs also recovers one-week post-OGD. Our analysis of mIPSCs recorded after one week showed recovery of IEI that is consistent with the gephyrin morphology (Fig. 5H-L; 235.1ms±27.24, n=15 versus 233.0ms±24.24, n=13, Two-tailed Unpaired T-test *P*=0.954). The cumulative probability distribution and the average values showed no difference in amplitude after one-week recovery (Fig. 5M-N; 35.77pA±2.48, n=15 versus 34.04pA±2.48, n=13; Two-tailed Unpaired T-test *P*=0.75).

It is worthy to point out that mIPSCs IEI values in control neurons 24 hr post recovery is significantly lower than IEI values after one-week recovery (233.0ms±24.24, n=13versus 146.0ms±10.25, n=15; Two-Tailed Unpaired T-test *P*=0.0037). This is surprising considering that the synapses within the hippocampal circuit mature between 3 to 4 weeks in culture and the IEI get usually smaller. Consistent with this idea, we also noticed a significant increase in the mean mIPSC amplitude in neurons with one-week recovery post-OGD (30.05pA±1.231, n=15 versus 34.04pA±2.48, n=13; Two-Tailed Unpaired T-test *P*=0.016).

BDNF transcription is upregulated post-OGD

Independent studies have reported that ischemia-induced increase in excitatory neurotransmission upregulates BDNF transcription and release [276, 297]. In order to test whether OGD resulted in a similar increase in BDNF in our experiments, we performed quantitative real time PCR (qRT-PCR) from control slices and OGD slices post-24 hr OGD recovery to measure changes in *bdnf* transcript levels from the CA1 and CA3 areas (Fig. 7A). Analysis upon normalization using the house-keeping gene GAPDH showed a significant increase in *bdnf* transcript specifically from the CA1 region (P=0.046) but not CA3 (P=0.14). This results confirms that exposing organotypic slice cultures to transient OGD causes a similar rise in BDNF levels as reported earlier.

BDNF facilitates structural changes at GABAergic synapses via gephyrin SUMOylation. Furthermore, it has been reported that there is a significant elevation of SUMO-1 and SUMO-2/3 proteins levels following ischemia [253]. Elevated levels in SUMO proteins could be a consequence of increased SUMO transcription; hence, we tested for *Sumo*-1 and *Sumo*-2 transcript changes following both 90 min and one-week post-OGD recovery. Quantification for mRNA levels showed no significant differences in *Sumo*-1 and *Sumo*-2 transcript levels between the control and OGD treated slices (Fig. 7B-C).

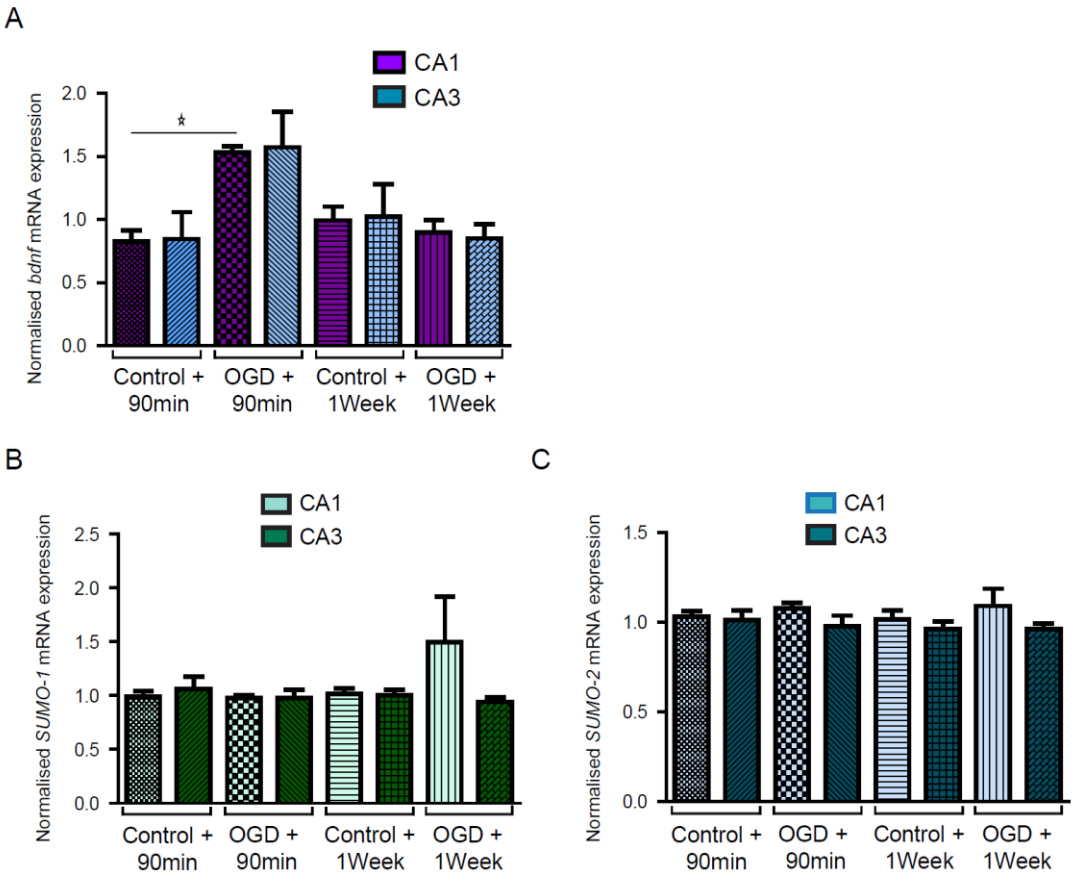


Figure 7: OGD results in increased BDNF transcript level.

(A) Real time qRT-PCR analysis of *bdnf* transcript in control and OGD samples 90 min and one-week post recovery. *$P<0.01$ Paired t-test **(B-C)** qRT-PCR analysis of *Sumo-1* and *Sumo-2* transcripts in control versus 90 min and one-week post-OGD recovery. The analyses were done using organotypic hippocampal slice cultures from 3 independent batches and a total of 5 slices for each condition. T-test showed no significant differences.

Blocking BDNF signaling prevents the decrease in GABAergic inhibition after OGD

Our data identifies elevated BDNF transcript levels after OGD. Furthermore, morphology analysis shows that scavenging BDNF using TrKB/Fc results prevents gephyrin cluster loss after OGD (Fig. 3). Hence, we wanted to test whether preservation of gephyrin clustering after scavenging BDNF also affected GABAergic inhibition. For this, we applied 1μg/mL of TrKB/Fc after OGD during the 24 hr recovery process and performed whole-cell patch-clamp recordings of GABAergic mIPSCs from CA1 pyramidal neurons.

We analyzed for changes in IEI and amplitude (Fig. 8A-C). In support of our earlier observations treatment of slices with TrkB/Fc to scavenge BDNF rescued GABAergic mIPSCs as we did not observe any significant difference in the IEI (Fig. 8D-E; 182.8ms±19.25, versus 141.9ms±15.49, Two-Tailed Unpaired T-test P=0.114). Furthermore, we also did not observe significant difference in the amplitude between control and TrKB/Fc treated slices after OGD (Fig. 8F-G; 28.63pA±3.02, n=11, versus 28.77pA±2.6, n=11, Two-Tailed Unpaired T-test *P=0.97*).

Our functional data is in line with the gephyrin morphology changes, wherein we show a direct correlation between BDNF upregulation, gephyrin cluster loss and increased IEI. Furthermore, by scavening BDNF using TrkB/Fc we demonstrate preservation of both gephyrin clusters and GABAergic mIPSCs.

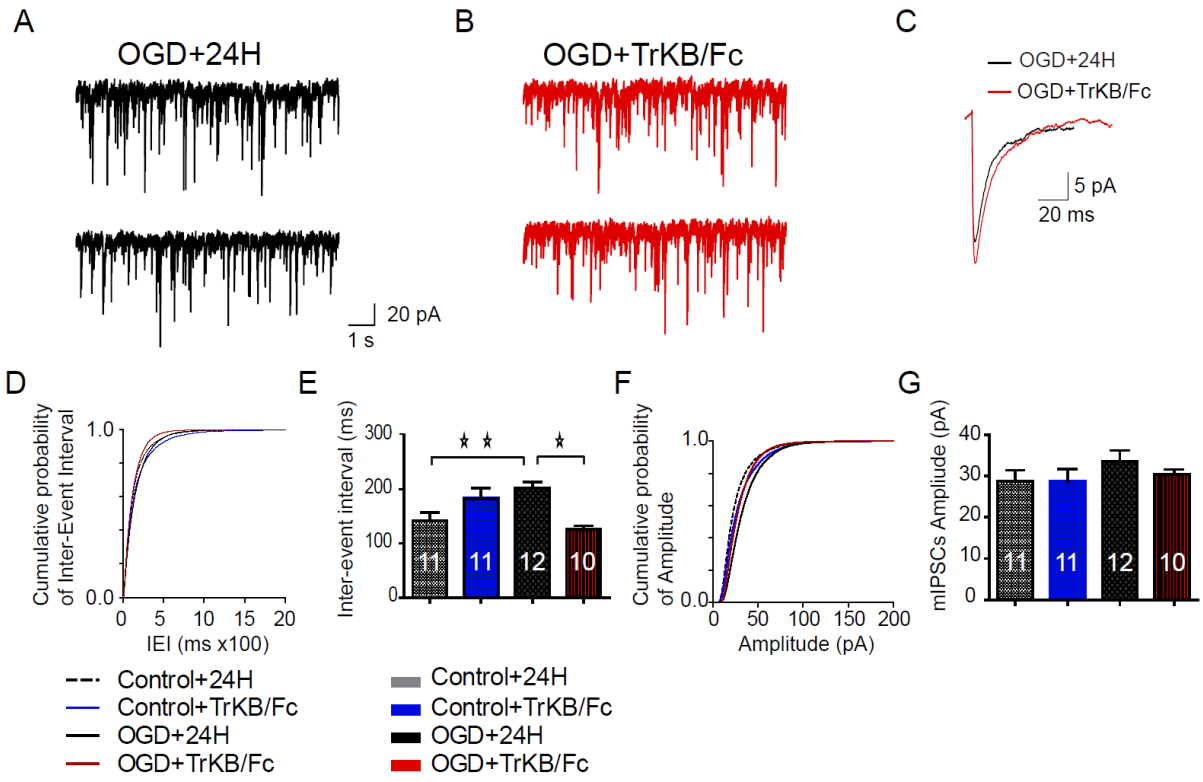
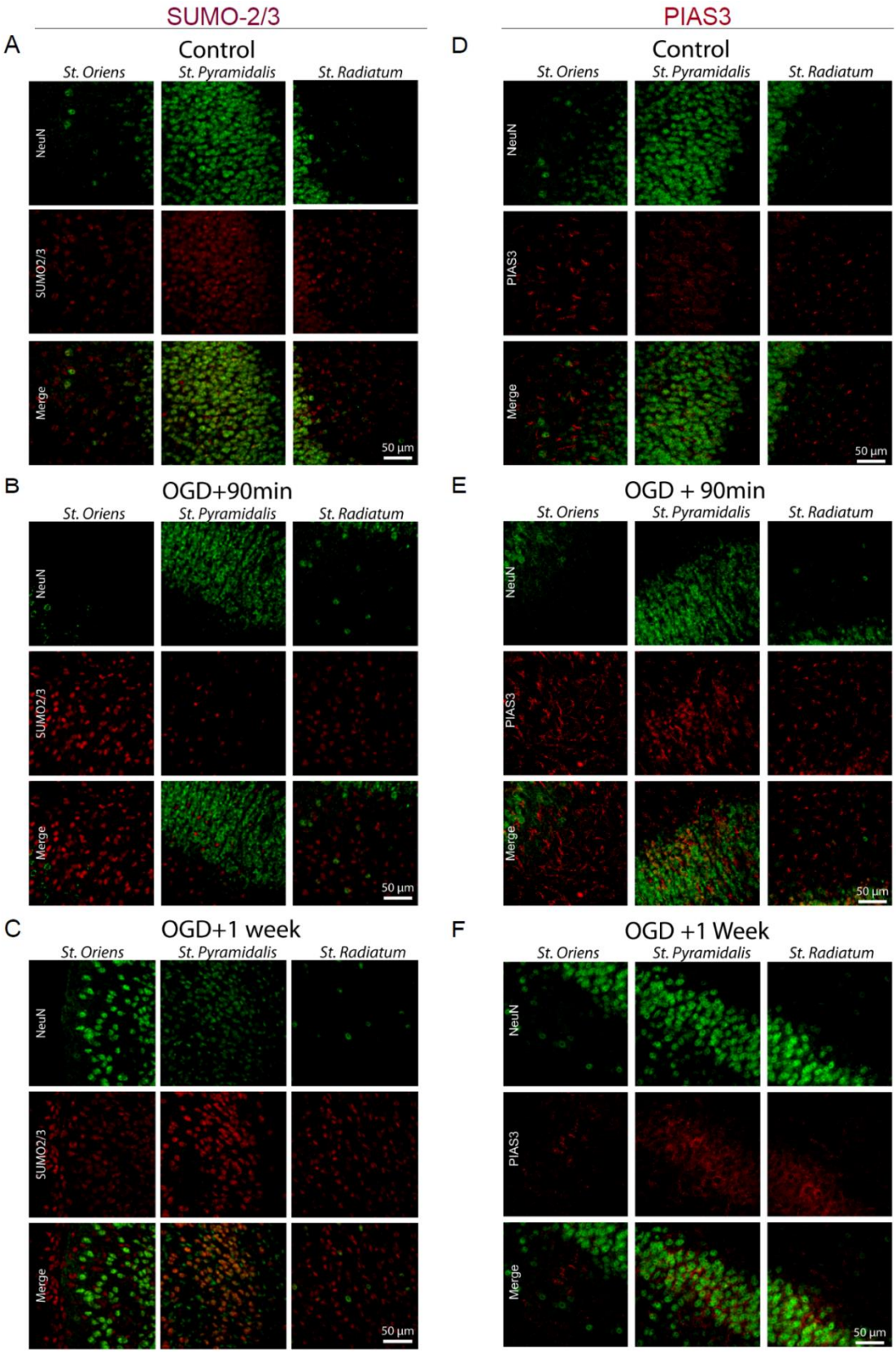


Figure 8: Scavenging BDNF rescues GABAergic mIPSC loss after OGD.

(A-B) Representative traces of GABAergic mIPSCs form DIV 21 CA1 pyramidal neurons 24 hr post-OGD (n=12) and cultures treated with TrKB/Fc (n=10). **(C)** Average traces of GABAergic mIPSCs. **(D)** Cumulative probability distribution of mIPSCs IEI: Control+24hr (n=11); Control+TrKB/Fc (n=11); OGD+24hr (n=12) and OGD+TrKB/Fc (n=10); ***P<0.01* KS test. **(E)** Quantification of mean mIPSCs IEI. ***P<0.001* One-Way ANOVA followed by a Tukeys Multicomparison test. **(F)** Cumulative probability distribution of mIPSCs amplitudes. **(G)** Quantification of mean mIPSCs amplitudes. Non-significant One-Way ANOVA followed by a Tukey s Multicomparison test.



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OGD alters the subcellular localisation of SUMO-2/3 and PIAS-3 in CA1 neurons

Our experiments in primary hippocampal neurons showed that BDNF alters subcellular localization of SUMO-1, -2/3 and PIAS-3 from nucleus to cytoplasm (Chapter 1). Independent reports have shown up regulation of SUMO-2/3 under ischemic conditions [253, 283]. We wondered if the observed loss of gephyrin clusters in OGD slice cultures is also accompanied by a subsequent subcellular localization change in SUMO-2/3 and PIAS-3 to influence gephyrin clustering at GABAergic synapse. In order to test this we stained for endogenous SUMO-2/3 and endogenous PIAS-3 (Fig. 9; in red) and morphologically analysed for distribution difference between different pyramidal cell layers in the CA1 area. In order to label the neurons we stained our slice cultures with the neuron specific nuclear marker protein NeuN (Fig. 9; in green). .

Under baseline conditions endogenous SUMO-2/3 staining showed a nuclear localization with a strong co-localization with NeuN (Fig. 9; yellow). This co-labeling was restricted to the *Stratum Pyramidale* where the pyramidal cell body is situated. However, in the *Stratum Oriens and Radiatum*, which contain the pyramidal cell dendrites we saw nuclear staining which we mostly attributed to the astrocytes. We could not observe any distinct dendritic labelling of the pyramidal cell neurons in *Oriens* or *Radiatum*.

Figure 9: OGD alters sub-cellular localisation of SUMO-2/3 and PIAS-3 in CA1 area.

(A-C) Organotypic slice cultures DIV 21 immunostained for endogenous SUMO-2/3 (red) and NeuN (green) within the three in *Stratum Radiatum*, *Stratum Pyramidalis* and *Stratum Oriens*, 90min post-OGD and one-week post-OGD. **(D-F)** Organotypic slice cultures DIV 21 immunostained for endogenous PIAS-3 (red) and NeuN (green). Localisation of endogenous PIAS-3 within the three different layers of hippocampal CA1 area; control condition, 90min post-OGD, one-week after OGD. Scale bar 50μm.

We then analysed slice cultures that had undergone OGD followed by either 90 min or one-week recover. At 90 min post-OGD, SUMO-2/3 showed a strong reduction in the *Stratum Pyramidale,* which, interestingly, recovered at one week post-OGD (Fig. 9B, D). Taken together, these results demonstrate that OGD alters the cellular localisation of endogenous SUMO-2/3 without affecting the transcript levels.

When we analysed for PIAS-3, we found immunoreactivity both in the nucleus and soma of NeuN positive cells in the *Stratum Pyramidalis* (Fig. 9D; yellow). However, we also found some immunoreactivity for non-neuronal cells (NeuN negative) in the *Stratum Oriens and Radiatum* (Fig. 9D). After OGD and 90 min recovery, PIAS-3 was localized differently; we found a strong staining in *Stratum Pyramidalis*, *Radiatum* and *Oriens* (Fig. 9E). Interestingly, after one-week post-OGD we observed that PIAS-3 staining in *Stratum Pyramidalis* returned to baseline while also showing elevated levels in *Stratum Radiatum* (Fig. 9F).

The localization difference of SUMO and PIAS-3 proteins within the neuronal dendrites in response to OGD is consistent with our observations in primary hippocampal neurons. Hence, we decided to evaluate and quantify specific morphological change in PIAS-3 in response to OGD. In order to achieve this we prepared organotypic slice cultures using transgenic mice that express myristoylated GFP in CA1 pyramidal neurons to facilitate the identification of dendritic structures at *Radiatum* and *Oriens* (Fig. 10A). We induced OGD and observed for subcellular distribution difference in PIAS-3 90 min and one-week later (Fig. 10A, B). We performed morphology analysis of the dendritic segments situated at the *Radiatum* and *Oriens* to quantify for PIAS-3 expression in apical and basal dendritic segments. We observed that PIAS-3 forms distinct puncta in the dendrites in addition to its nuclear labelling (Fig. 10A; zoom). Hence, we quantified for PIAS-3 cluster area and density change in response to OGD.

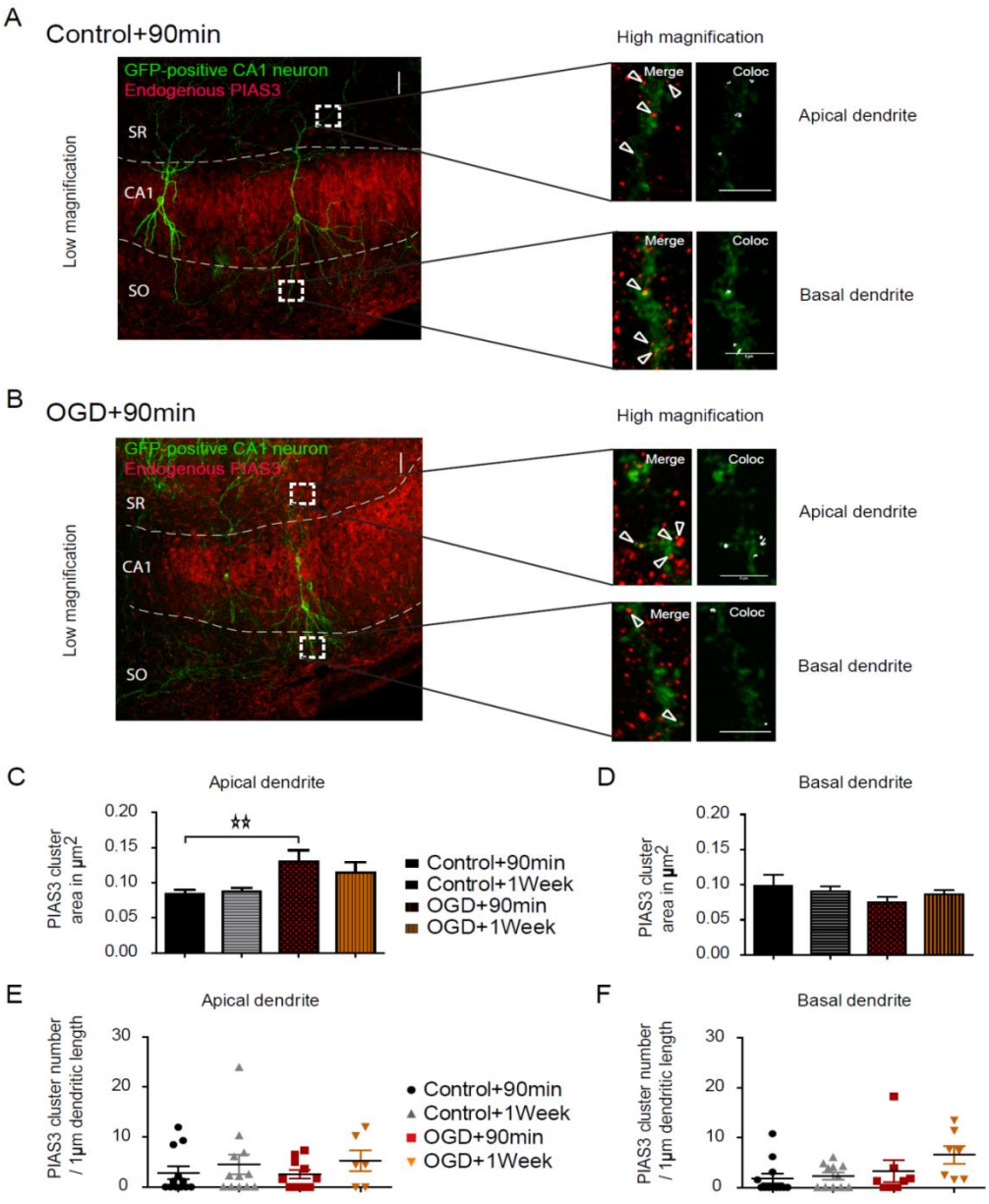

Figure 10: PIAS-3 dendritic puncta is altered in response to OGD.

(A-B) Organotypic slice culture of CA1 pyramidal neurons expressing GFP stained for PIAS-3 (red), in Control+90min and OGD+90min slices. Different CA1 layers are indicated; SR *Stratum Radiatum*; CA1 *Cornu Ammonis* and SO *Stratum Oriens*. Scale bar 50µm. High magnification image is on the right side show PIAS-3 sub-cellular localisation in the apical and basal dendrites. Arrow show co-localised PIAS-3 on CA1 pyramidal neuron dendrite. Scale bar 5µm. **(C-D)** Quantification of PIAS-3 puncta size within the apical and basal dendrites (***P*<0.01 KS test). **(E-F)** Quantifications of PIAS-3 puncta number per 1µm length dendrite (non-significant One-way ANOVA).

Apical dendritic segments showed an increase in PIAS-3 puncta size after OGD (Fig. 10C; 0.13μm²±0.016 versus 0.084μm²±0.006, KS test *P*=0.003). Interestingly, PIAS-3 puncta size was still significantly larger one-week after OGD (Fig. 10C; 0.11μm²±0.015 versus 0.13μm²±0.016, KS test *P*=0.072). We did not see any significant change in the PIAS-3 puncta size on the basal dendrite (Fig. 10D; 0.11μm²±0.015 versus 0.088μm²±0.005, KS test *P*=0.24).

The density of PIAS-3 puncta remains unchanged in the apical dendrites (Fig. 10E; One-Way ANOVA $F_{(3,36)}$=0.54, *P*=0.66) and in the basal dendrites (Fig. 10F; One-Way ANOVA $F_{(3,35)}$=0.11, *P*=0.196) independently of any of the conditions. Interestingly, PIAS-3 puncta size is significantly increased in the apical dendrites condition (Fig. 10D; One-Way ANOVA *P*=0.0058). In contrary, PIAS-3 puncta size in the basal dendrites is unaffected regardless of the condition (Fig. 10D; One-Way ANOVA *P*=0.18). Thus, the main change is occurring at *Stratum Radiatum* where pyramidal CA1 neurons receive synaptic contacts from the CA3 mossy fibres.

Gephyrin SUMO mutations prevent impaired postsynaptic clustering after OGD

We have demonstrated that SUMOylation exerts bidirectional effects on gephyrin clustering, likely through the involvement of PIAS-3 and SENP-2/-6 to facilitate efficient SUMO conjugation and de-conjugation [Ghosh et al., submitted]. Furthermore, many independent studies have shown gephyrin scaffold regulation having direct implications on GABAergic transmission. Our results so far implicate ischemia-induced reduction in GABAergic transmission to gephyrin cluster loss. Moreover, our results also identify distinct effect of ischemia on subcellular localization difference for PIAS-3 and SUMO-2/-3. Taking together these observations, it is likely that ischemia-induced alterations at GABAergic synapse are facilitated by changes in gephyrin SUMOylation. We have recently identified and characterized SUMO-1 (K148) and SUMO-2 (K724) conjugation sites on gephyrin. In primary neuron cultures, mutating these two SUMO conjugation sites via site directed mutagenesis stabilizes GABAergic synapses by rendering them insensitive to BDNF modulation. Hence, we decided to test whether expression of eGFP-K148R/K724R double mutation would make gephyrin resistant to ischemia-induced alterations and prevent changes in postsynaptic clustering. We transfected mCherry-

gephyrin or mCherry-K148R/K724R plasmids into organotypic slice cultures at DIV 14 and induced ischemia at DIV 16 (Fig. 11). Analysis for changes in eGFP-gephyrin clustering (Fig. 11A) showed a significant reduction in cluster volume (Fig. 11C; 0.03µm^3±0.002 versus 0.019µm^3±0.0018, KS test P<0.001) and density (Fig. 11D; 0.65±0.01/µm versus 0.33±0.03; P=0.001) at 90 min post-recovery. Interestingly, analysis for mCherry-K148R/K724R (Fig. 11B) cluster volume (Fig. 11C; 0.03µm^3±0.005 versus 0.046µm^3±0.003, KS test P=0.116) and density (Fig. 11D; 0.75±0.15 versus 0.82±0.07; P=0.93) showed no change at OGD+90min. These observations confirm our idea that ischemia induced plasticity change at GABAergic postsynapse is primarility facilitated by PIAS-3 induced SUMO-conjugation on gephyrin at K148 and K724 residues.

Overall, our data demonstrates the significance of gephyrin SUMOylation in downregulating GABAergic transmission after OGD.

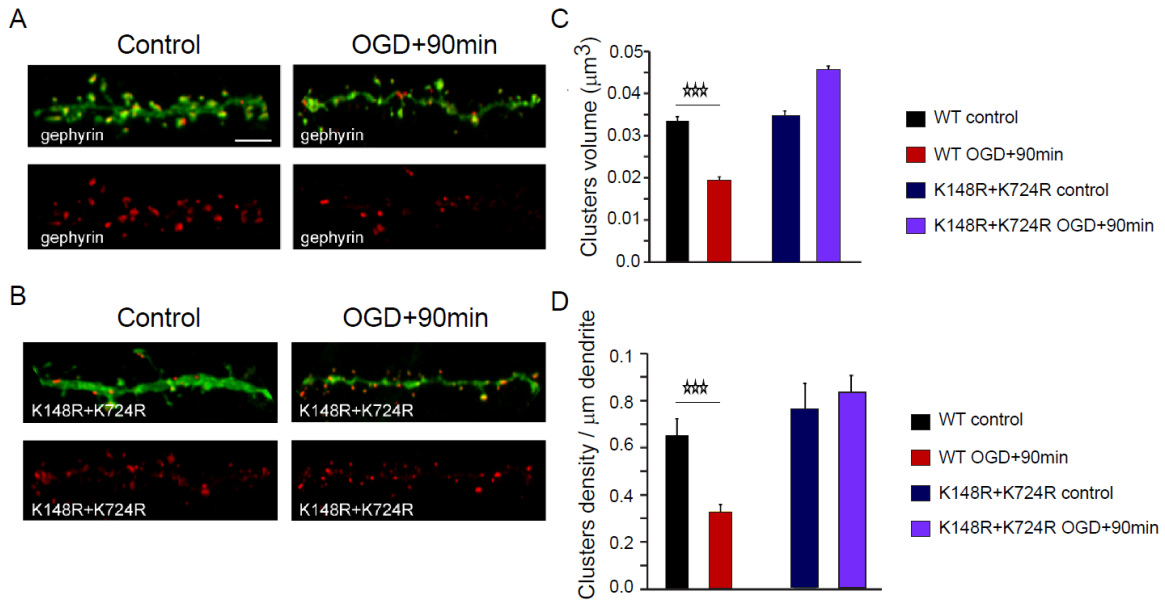

Figure 11: Gephyrin SUMO-deficient mutant prevents impaired postsynaptic clustering after OGD. **(A-B)** DIV 14 hippocampal organotypic cultures co-transfected with td-Tomato and either WT gephyrin or gephyrin SUMO-deficient mutant eGFP-K148R+K724R (red puncta) using Gene Gun technique. Scale bar 3µm. **(C-D)** Quantifications of gephyrin mean puncta volume and density per µm dendritic length in control condition and OGD+90min. ***P<0.001 Student t-test.

Discussion

In this study, we use organotypic hippocampal slice cultures and an *in vitro* model of ischemia, oxygen glucose deprevation (OGD) to investigate the role of BDNF-induced gephyrin SUMOylation for GABAergic synaptic plasticityin CA1 pyramidal neurons. We demonstrate that after OGD there is a specific elevation of BDNF transcription. Further, we identify SUMOylation downstream of TrkB signaling as the mechanism responsible for the transient loss of gephyrin clusters after OGD. By expressing SUMO-insensitive gephyrin mutants, we provide proof of principle for this mechanism of ischemia-induced impairment of gephyrin scaffolding loss at GABAergic terminals, resulting in reduced GABAergic transmission.

OGD effects on gephyrin scaffolding and inhibitory GABAergic transmission

We present data showing disruption in gephyrin scaffolding and GABAergic mIPSC frequency in response to OGD. Although we see a 90% decrease in gephyrin expression after OGD, the reduction in GABAergic mIPSCs frequency is only 35%. Furthermore, we show a 20 to 30% reduction in the expression of α1 and α2 subunits-containing $GABA_A$Rs after OGD. Quantitative nanoscopy measurements have shown that the stoichiometry of gephyrin to $GABA_A$R is not 1:1, indeed it is likely to be 5:1 [132, 298]. This is based on the fact that each gephyrin molecule can bind multiple $GABA_A$R subunits within a receptor. Direct interaction between gephyrin and $GABA_A$R α_{1-3}, β_{2-3} and γ2 subunits has been reported [18, 130, 136], and usually 2α, 2β and 1γ/δ/ε subunits assemble together to form a pentameric ligand gated channel [20]. This could explain partially the higher loss of gephyrin expression compare to the $GABA_A$R subunits.

In recent years it has become evident that gephyrin cluster detection using the most commonly used monoclonal antibody (m7a) is influenced by gephyrin post-translational modification, especially phosphorylation. Phosphorylation at S270 residue renders the gephyrin clusters insensitive to m7a detection in neurons. Hence, another more simplistic explanation could be that OGD induced post-translational modification(s) on gephyrin could make morphological detection of endogenous gephyrin clusters difficult leading to a more dramatic decrease in cluster numbers compared to $GABA_A$Rs.

BDNF and SUMO pathway regulate gephyrin scaffolding after OGD

Here, we inhibit OGD induced loss of GABAergic synapse by scavenging BDNF. It has been shown that neurotrophin NT-4 signaling can also reduce brain injury after stroke [299]. We eliminate the involvement of NT-4 in our ischemia model because in an independent study we identify a specific role for mature/processed BDNF for facilitating change at GABAergic synapse (Gill et al. in preparation). In this study, we could prevent OGD induced gephyrin cluster loss by blocking mature BDNF signaling using the monoclonal antibody N9. Given this evidence it is likely that OGD mediated downregulation of gephyrin scaffolding in response to OGD is via the neurotrophin BDNF and not NT-4.

We have demonstrated that BDNF regulates specific proteins of the SUMO pathway to influence gephyrin scaffolding property at GABAergic synapse (see Chapter 1). We also demonstrate the specificity of BDNF induced sub-cellular localization change of SUMO E3 ligase PIAS-3, and its subsequent influence on gephyrin scaffolding. Consistent with our earlier observations in primary neuron culture, our data using organotypic hippocampal slice culture also proves OGD induced sub-cellular localization change of PIAS-3 (Fig. 10). Interestingly, we do not observe any transcriptional change in *PIAS-3* or *Sumo* transcripts, suggesting that OGD affects SUMO pathways signaling rather than SUMO availability and/or conjugation. In further support of this idea, we have seen BDNF induced sub-cellular localization changes for SENP-2/-6 (See Chapter 1). Hence, it is possible that spatial and temporal availability of PIAS-3, SUMOs and SENP-2/-6 influence gephyrin SUMOylation levels in response to OGD. It has been demonstrated that gephyrin is a novel SUMO substrate and is modified by SUMO-1 and SUMO-2 at K148 and K724 residues respectively [Ghosh et al., submitted]. Here, we build on this evidence and provide proof of principle by showing complete blockade of gephyrin cluster loss after OGD upon expression of SUMO-defective gephyrin mutants K148R/K724R in CA1 neurons (Fig. 11).

Role of BDNF and SUMOylation for recovery after an ischemic injury

In an independent study we investigated the importance of gephyrin phosphorylation by ERK1/2 and GSK3β kinase pathways for gephyrin scaffold stabilisation at GABAergic synapses in response to OGD. In this study, we identified that BDNF activates ERK1/2 and GSK3β pathways causing phosphorylation at gephyrin S268 and S270 residues resulting in proteolytic clipping of gephyrin scaffold by the protease calpain1 [Gill et al., in preparation]. This loss of gephyrin scaffold could provide a molecular basis for rapid removal of GABAergic synapses in CA1 pyramidal neurons after OGD. Our observations of gephyrin degradation and removal from GABAergic synaptic sites is supported by independent report showing MCAO induced gephyrin loss via calpain1 activity [248]. We could successfully reverse OGD induced gephyrin cluster loss by the expression of gephyrin S268A/S270A phosphorylation defective mutations in CA1 pyramidal neuron [Gill et al., in preparation].

Given that gephyrin SUMO defective mutations enhance scaffolding at GABAergic synapses, it is likely that SUMO conjugation restricts gephyrin scaffolding properties [Ghosh et al., submitted]. We could also demonstrate that SUMOylation, phosphorylation and acetylation pathways cross-talk to influence gephyrin scaffolding. Furthermore, using an animal model lacking *Gabra2* (α2 $GABA_AR$ subunit), wherein we see a distinct loss of gephyrin scaffolding, we could successfully rescue gephyrin scaffolds via *in vivo* transgenic expression of eGFP-K148R SUMO-defective mutation [Ghosh et al., submitted]. The successful rescue of gephyrin scaffold using the SUMO defective mutation suggested to us that SUMO pathway operates upstream of the phosphorylation pathway for gephyrin cluster regulation. In support of this interpretation, it has been reported that ERK 1/2 expression and activity undergo a long-lasting decrease during the recovery phase post-ischemia [300]. Hence, we can postulate that gephyrin SUMOylation prevents reestablishment of GABAergic synapses in the immediate aftermath of OGD. However, during the recovery phase, SENP-2/-6 mediated SUMO de-conjugation, along with suppressed ERK1/2 kinase activity facilitates gephyrin scaffold building and establishment of GABAergic inhibition, thus facilitating network activity.

Our idea is supported by observations from other groups showing a rapid and long-lasting increase in general SUMOylation levels, after global or focal middle cerebral artery occlusion (MCAO) [242, 253]. Further, another study shows a distinct correlation between inhibition of ERK activation and an elevation of GABAergic transmission after OGD [300]. Studies have also proposed a neuroprotective role for substrate SUMOylation post ischemic stress [242] [241]. Although transient focal ischemia in rodents also induces elevation in protein SUMOylation levels [242, 253, 301], no clear proof for neuroprotection has been demonstrated. One study using SUMO overexpression in SHSY5Y human neuroblastoma cells and in rat cortical neurons demonstrate resistance of the cells to OGD [302].

The increase in the general SUMO conjugated proteins has been proposed to be beneficial at glutamatergic synapses by regulating the level glutamate receptors at synaptic sites [190, 221]. It has been shown that under physiological conditions kainate receptor GluR6 [226] is a SUMO substrate. GluR6 is abundantly expressed in the hippocampus and has been shown to be endocytosed after SUMOylation. This supports the idea of a possible downregulation of neuronal excitation following ischemic injury via the process of SUMOylation [220].

Multiple studies show a beneficial role of BDNF for lowering the infarct volume or protecting against cellular damage [303, 304] or for motor recovery in models of stroke [305, 306]. In contrary, other studies have also shown the benefit of low BDNF levels for motor recovery, in the aftermath of stroke [307, 308]. Our study resolves some of these discrepancies in literature by demonstrating different effects for acute and chronic BDNF signaling.

Overall, our study sheds light into some of the long-standing issues in the field of ischemia, such as what is the relationship between BDNF and GABAergic synapse loss. We dissect the molecular mechanism by linking BDNF signaling with gephyrin SUMOylation for GABAergic synaptic plasticity. This study offers a better understanding of the complexity underlying BDNF signaling during recovery following ischemic injury.

IV/ General discussion

In this thesis, we demonstrate the importance of gephyrin regulation by PTM for its scaffolding at GABAergic postsynaptic sites and for the regulation of GABAergic IPSCs under patho-physiological conditions. Most prominent among these PTMs is SUMOylation, a recently identified modulator of postsynaptic gephyrin clustering. Moreover, we identified BDNF as an upstream signaling molecule activating the SUMO pathway to regulate gephyrin cluster formation and maintenance. The relevance of this mechanism has been tested in a model of ischemia, where BDNF and SUMOylation are considered to provide neuroprotective signaling for recovery post-injury. Herein, I will summarize and discuss the main findings of this thesis.

In our previous report [Ghosh et al. Submitted] and in Chapter 1, we present supportive data identifying the E3 ligase PIAS-3, as a key player of the dynamic modulation of gephyrin clustering at GABAergic postsynaptic sites. PIAS-3 regulates gephyrin clustering via two independent mechanisms involving a SUMO and a non-SUMO ligase function. Both mechanisms are under the control of the BDNF signaling. We demonstrate that acute BDNF exposure modulates gephyrin clustering via SUMOylation in primary hippocampal neurons. Direct evidence for gephyrin SUMOylation is provided by the inhibition of BDNF effect in cells overexpressing the SUMO-deficient gephyrin mutant constructs (eGFP-K148R/K724R). BDNF signaling inhibits PIAS-3 function and activates at the same time the kinases ERK1/2 and GSK3β pathways. As a result, gephyrin becomes less SUMOylated and becomes more phosphorylated by these kinases, leading presumably to gephyrin cleavage (smaller clusters). Conversely, gephyrin phosphorylation by ERK1/2 and GSK3β regulate its SUMOylation propensity, providing a mechanism to rapidly adjust cluster size and density in response to extracellular signals.

Recent reviews highlight the possibility that SUMOylation PTM might be a key component of recovery post-ischemic injury *in vitro* and *in vivo* [220, 234, 240, 242]. Likewise, there is strong evidence for the importance of BDNF after stroke [278, 304, 305, 309]. We observed in the OGD model of ischemia (Chapter 2) a profound reduction of gephyrin and $GABA_A$Rs-containing α1

and α2 subunits clusters, accompanied by a downregulation of GABAergic transmission. These results are in line with previous studies [248]. Furthermore, these effects depend on BDNF signaling, as we could prevent them via scavenging BDNF by TrKB-Fc. Finally, gephyrin cluster loss post-OGD could be prevented via over-expressing the SUMO-deficient gephyrin mutant constructs. Taken together, we here provided a mechanism downstream of BDNF in the regulation of GABAergic synapse plasticity post-ischemic injury. It is tempting to speculate that it relies on the same signaling pathways as those uncovered in primary neurons. Moreover, based on BDNF negative modulation of GABAergic synapse function and structure, the data highly suggest a non-protective role of BDNF post-recovery.

Ischemia and inhibitory transmission

The importance of inhibitory neurotransmission for the maintenance of network activity might be even greater when studying a disease, such as stroke, than under baseline conditions. The hippocampus is a brain region highly vulnerable to ischemia [279, 310]. Cerebral ischemic stroke induces an early disruption of GABAergic transmission via dysregulating different component necessary in the maintenance and the proper function of GABAergic synapses. One study has demonstrated the downregulation of CA1 interneurons excitability following transient ischemia. This decrease in general excitability could be explained by a decrease in potassium channels $NA_v1.1$ expression, which might suggest a reduction in GABA release. This voltage-gated channel play an essential role in the generation and propagation of action potential [311]. Intriguingly, impairment of inhibition would enhance pyramidal neurons excitability thereby facilitating NMDA excitotoxicity and contributing to neuronal cell death. Otherwise, in general, studies on ischemia report a general downregulation of GABAergic transmission occurring at the pre- and postsynaptic levels [248, 284].

The studies that have been reporting some changes at GABAergic presynaptic terminals are controversial, because their conclusions vary with the ischemic paradigm used. One study reported a decrease in vesicular transporter vGAT following brain ischemia [312], while another one demonstrated a resistance (no change) of vGAT to different type of brain ischemia [313].

There is agreement, however, that glutamic acid decarboxylase (GAD), the enzyme responsible for GABA synthesis, is unaffected by any type of brain ischemia [314].

Postsynaptically, a transient or focal ischemic injury leads to a decrease in $GABA_A$Rs protein expression [284, 290, 315] and in $GABA_A$Rs subunits mRNA levels [314], causing a general decrease in GABA-mediated responses [316]. Interestingly, an independent study has reported the downregulation of $GABA_A$Rs and gephyrin to be a calpain-dependent mechanism. In addition, these authors demonstrated that the receptors undergo dephosphorylation by PP1α/PP2A, in turn facilitating their endocytosis [248]. Moreover, this downregulation of $GABA_A$Rs might also be the consequence of the reduction in the interaction between the receptors and gephyrin [252]. The loss of this interaction might contribute to the acceleration of $GABA_A$Rs internalization. Gephyrin is important for trapping the receptors at the synapse, and without this interaction the receptors are more mobile at plasma membrane [87, 131, 136].

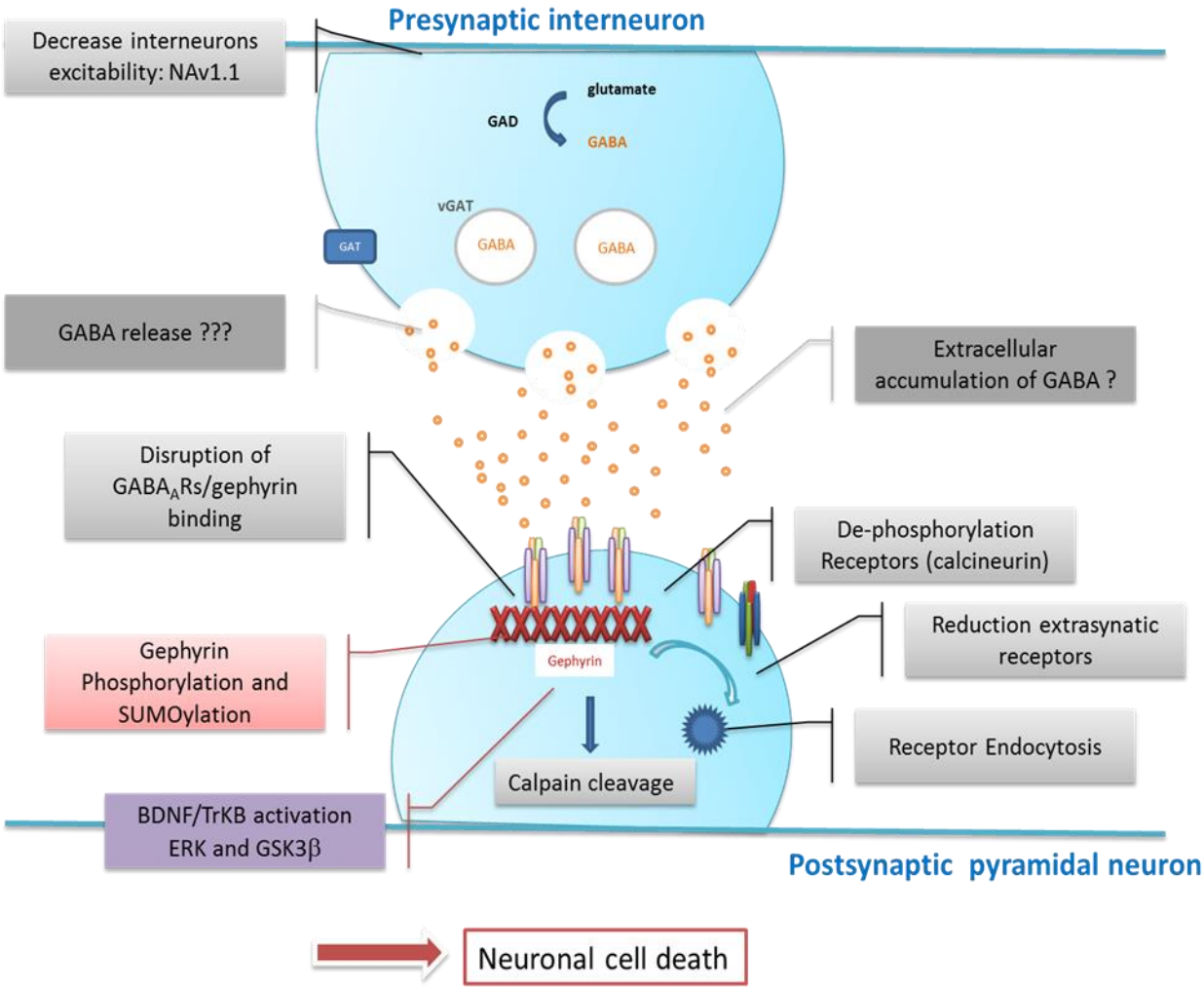

Figure Summary: consequences of ischemia on inhibitory synapses.

In grey are stated the fact described within the literature. A question mark highlights the non-confirmed findings. In red and purples are the results obtained and presented in this thesis.

Our work provides strong support to the notion that regulation of gephyrin PTM is a key component in the reduction of GABAergic function upon stroke. Therefore, show that BDNF release might paradoxically aggravate the effects of ischemia by directly down-regulating GABAergic transmission. Furthermore, we demonstrated in Chapter 1 that gephyrin SUMOylation cross-talks with gephyrin phosphorylation. Intrigued by these results, we have used the gephyrin phospho-deficient mutant (eGFP-S268A/S270A) in organotypic slice cultures and observed its resistance to OGD [Gill et al. Unpublished]. Therefore, both PTMs participate in the downregulation of gephyrin post-OGD and their inhibition would maybe contribute in the

maintenance of GABAergic function. Thus, the cross-talk between phosphorylation and SUMOylation is a striking example of the dynamic modulation of gephyrin, and thereby GABAergic synapse plasticity, under patho-physiological conditions (Figure Summary).

Further, these data provide some support to the idea that stabilization of $GABA_A$Rs surface expression would be neuroprotective in neurons subjected to ischemic injury [315]. Moreover, it has been shown that enhancing GABAergic transmission, using benzodiazepines, would prevent neuronal cell death right after ischemia, in adult hippocampus [247].

It would be of great interest to study whether the mechanisms described in this thesis also hold under *in vivo* ischemic stroke, such as studied by transient middle cerebral artery occlusion (MCAO). It is conceivable that stabilizing gephyrin would help retaining $GABA_A$Rs in the PSD, contributing in maintaining GABAergic transmission. Thereby, enhancing inhibition of pyramidal neurons would help decreasing excitotoxicity.

To test this possibility, it would be possible to overexpress gephyrin SUMO-deficient mutant constructs by transfection with an AAV virus injected directly in the brain region of interest. After leaving enough time for the virus to be expressed, the animal model would undergo MCAO treatment followed by 24h recovery. As we are interested in GABAergic synapses, it would be then interested to look if the infected target neurons would show resistant to MCAO against downregulation of gephyrin expression like obtained *in vitro* (Chapter 2). Addition staining of different markers at GABAergic synapses such as vGAT and $GABA_A$Rs subunits, would be a support in the idea that gephyrin prevention would contribute in keeping the receptors at the synapse and so, prevent there endocytosis. Moreover, as it was speculated in the literature, it would be interesting to see whether the modulation of GABAergic synaptic transmission would contribute in the prevention of neuronal death. Morphological changes are always accompanied by functional changes; it would be of great interest to test whether over-expressing this gephyrin mutant would contribute in the maintenance of GABAergic synapse transmission. If gephyrin get stable in those infected cells, leading to the stabilization of $GABA_A$Rs, it would be therefore expected to have prevented the downregulation of GABAergic mIPSCs post-ischemia. This would be possible to study via performing patch-clamp recording of CA1 pyramidal neurons of hippocampal slices made 24h-48h post-reperfusion. This experiment would also allow seeing, in

case of conservation of GABAergic synapses, the possible changes at glutamatergic synapses. In parallel, NMDA-mediated excitatory transmission would also be recorded in order to see if prevention of decrease inhibitory transmission would decrease glutamatergic excitability.

However, ischemia induces a variety of molecular and cellular consequences that it would be possible that glutamatergic transmission might not be totally rescued or maintained in a steady-state by only acting at GABAergic levels. Others cells such as glial cells have also been reported to be affected by ischemia. Microglia, oligodendrocytes and astrocytes play a crucial role in regeneration, survival and protecting brain function but also destroying in pathological conditions. The role of astrocytes in cerebral ischemia is quiet controversial and still need to be defined. So many questions have been raised since the last decade which can't discriminate astrocytes from being neuroprotective or contributors to stroke injury [317]. It would be therefore interesting to look at their reactivity and see if they still, for example, lead to reactive gliosis even inhibitory synapses are more stable. Astrocytes have the capacity to limit the infarct size via the formation of the gliosis. Moreover, knowing that neurons can't survive without glia cells, it would be of great interest to determine their survival rate in comparison to the neuronal loss. Oligodendrocytes died when deprived of substances leading to neuronal disruption [317].

Thus, glial cells are crucial components to take in consideration when studying ischemia.

SUMOylation and ischemic preconditioning

In comparison to permanent or global, transient ischemia is known to induce non-lethal and smaller infarct size. Interestingly, ischemic preconditioning is the ability to have or induce very shorts ischemia, limiting the infarct size. A short ischemia seems to activate neuroprotective mechanisms important against a second ischemic insult which can be stronger. In the different independent studies made around neuroprotective effects, occurring following a short ischemic insult, point out the presence of an elevation of the general SUMO levels [253, 283, 318]. Moreover, it has been shown that over-expressing the different SUMOs paralogs *in vitro*, would contribute to the resistance of the primary neuronal cells to the ischemic insults such as OGD

[234, 241, 242]. Silencing SUMO-2/3, using lentiviral microRNA, facilitates neuronal death [241].

Therefore, SUMOylation might participate in inducing ischemic tolerance. However, further work would be needed in order to identify the targets SUMO substrates responsible of this protective role of SUMOylation. Regarding our findings, our results presented in this thesis could provide a mechanism of regulation at GABAergic synapses after a non-harmful ischemic injury.

It would be of great interest to see whether downregulating gephyrin clusters at GABAergic post-synapses is part of the neuroprotective process. The use of gephyrin SUMO-deficient mutants expressed in cell or slices cultures would either or not be beneficial for make the cells resistant to OGD.

Moreover, as highlighted previously, stabilizing GABA might not be sufficient to improve neurological function after stroke as many others components or cells are also involved in neuronal degeneration. Thus, it would be interesting to study the role of astroglia cells and microglia in addition at observing the different changes that would gender on glutamatergic synapses.

For recovery, the neuronal network might need therapeutics interventions on the three main cells: GABAergic and glutamatergic neurons in addition to glial cells. All three are affected by cerebral ischemia and all three are playing different role which still need to be defined. However, it can be believed that acting on one of those cells can be helpful for recovery or prevention of certain cerebral damage but not be sufficient for full recovery.

V/ References

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VI/ Abbreviations

ANOVA	Analysis of variance
AP2	clathrin-adaptor protein
ATP	Adenosine 5’ tri phosphate
BDNF	Brain-Derived Neurotrophic Factor
BZ	Benzodiazepine
CA1 or CA3	*Cornum Amonis* 1 or 3
CamK	Calmodulin Kinase
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CASK	Ca^{2+}/calmodulin-dependent serine protein kinase
CB	Collybistin
CCK	Colecystokinin
CNS	Central Nervous System
CREB	cAMP-responsive element binding protein
DIV	Days in vitro
Dlc	Dynein light chains
DMSO	dimethyl sulfoxide
eGFP	Enhanced Green Fluorescent Protein
ERK	Extracellular signal-Regulated kinase
FBS	Fetal Bovine serum
FCS	Fetal Calf serum
GABA	γ Amino Butyric Acid
$GABA_ARs$	γ Amino Butyric Acid type A Receptors
GABARAP	$GABA_AR$-associated protein
GAD	glutamic acid decarboxylase
GAT	GABA transporter
GFP	Green fluorescent protein
GSK3β	Glucogen Synthase Kinase 3β

HEK	Human Embryonic Kidney cells
HIF1α	Hypoxic inducible factor 1α
IEI	Inter-event interval
IP	Immunoprecipitation
JAK	Janus kinase
KD	Knock-down
KO	Knockout
KS	Kolmogorov-Smirnov
LTD	Long-term depression
LTP	Long-term potential
MAPK	Mitogen-activated protein kinase
MEF2A	myocyte enhancer factor 2A
Mena/VASP	Mammalian enabled/vasodilator stimulated phosphoprotein
MITF	Microphtalmia Transcritption Factor
mIPSCs	miniatures Inhibitory Postsynaptic Currents
mGluR	G-protein coupled metabotropic glutamate receptor
MOCO	Molybdenum cofactor
NL	Neuroligin
NT	Neurotrophins
OGD	Oxygen and Glucose deprivation
$P75^{NTR}$	p75 Pan Neurotrophin
PEI	Polyethylenimine
PI	propidium iodide
PI3K	Phophoinositide 3-Kinase
PIAS	Protein Inhibitor of Activated STAT
Pin1	Peptidyl-prolyl isomerase NIMA interacting protein 1
PINIT	Pro-Ile-Asn-Ile-Thr
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phopholipase C
PP1α	Protein phosphatase 1 alpha

PP2A	Protein phosphatase 2A
PSD	Postsynaptic density
PRIP1/2	phospholipase C-related catalytically inactive proteins 1/2
proBDNF	Precursor BDNF
PTM	Post-translational modification
PV	Parvalbumin
SAP	Scaffold Attachement factor-A/B
SDS	Sodium Dodecyl Sulfate
SENP	SUMO Sentrines Proteases
SIM	SUMO-interacting motif
SLM	*Stratum Lacunosum-Moleculare*
SO	*Stratum Oriens*
SR	*Stratum Radiatum*
STAT	Signal transducer of Activated transcription
STUbls	SUMO-targeted ubiquitin E3 ligase
SUMO	Small Ubiquitin like modifier
TBS	Tris Buffer Saline
TrKB	Tropomyosin-related Kinase B
TTX	Tetrodotoxin
Ubc9	Ubiquitin-conjugating enzyme 9
vGAT	Vesicular GABA transporter
WB	Western Blot
WT	Wild Type

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PROFESSIONAL EXPERIENCE

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Title*: Vasopressin excites mouse OVLT neurons via activation of Trpv1 channels.*

Supervised by *Dr.* ***Charles W. BOURQUE,*** Centre for Research in Neurosciences at the Montreal General Hospital, McGill University, Montréal, Canada.

April-Jun 2010: **Master Project I**

Title*: Electrical activities of Magnocellular Neurons from Rat's Hypothalamus.*

Dr. Stéphane OLIET "Glie-Neurone Relations" at the François Magendie Neurocenter from Bordeaux University (France).

Master 1 Project supervised by ***Dr. Jean-Marc ISRAEL***.

May 2009: **Bachelors Project**

Title: *Axonal guidance of Dopaminergic Neurons from Ventral Midbrain by eA5.*

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Teaching

Spring term 2013 and 2015	BIO 405 block course of Human Biology Module, University of Zürich

PUBLICATIONS in preparation

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1. Ghosh H., Auguadri L., Battaglia S., **Thirouin Z.S.,** Messner S., Acuña M., Wildner H., Yévenes G.E., Dieter A., Kawasaki H., Hottiger M., Zeilhofer H.U., Fritschy J.-M., Tyagarajan S. K.. Concerted action of SUMOylation, acetylation and phosphorylation cascades regulate gephyrin scaffolding and GABAergic transmission. (Submitted).

2. Gill R., **Thirouin Z.S**., Tyagarajan S.K., McKinney R.A. BDNF regulates synapse maintenance in hippocampal CA1 pyramidal neurons after oxygen glucose deprivation.(in submission)

3. **Thirouin Z.S**., Gill R., Früh S., .J-M. Fritschy, McKinney R.A., Tyagarajan S. BDNF regulates PIAS-3 function for modulating gephyrin formation at GABAergic synapses.

4. **Thirouin Z.S.,** Gill R., P.K.-Y. Chang, McKinney R.A., Tyagarajan S. BDNF regulates GABAergic synapse maintenance after OGD via gephyrin SUMOylation in CA1 hippocampal neurons.

5. Gizowski, C., **Thirouin, Z.S.,** Trudel., E., Zaelzer, C., Bourque, C.W. Clock neurons mediate anticipatory thirst prior to sleep. (in preparation)

Abstract for TALK

ZNZ PhD. retreat 2013 *(April 25 to 2, Valens Clinic, Switzerland.)***:** "BDNF mediated changes at GABAergic synapses"

Abstract for POSTERS

2012: Pharmocological Poster Day organized by UZH and ETH universities of Zurich (October 4th)**.** Poser presentation: ***Molecular Mechanism of Ischemia- Induces Changes at GABAergic Synapse.*** Z.S. Thirouin, R.Gill, L. Auguardi, A. Mc Kinney, S. Tyagarajan.

2013:

17th Annual meeting of the Swiss Society of Neurosciences *(February-2nd, Geneva, Switzerland)* Poster presentation: ***Molecular Mechanism of Ischemic Induces Changes at GABAergic Synapse*** Z. S.Thirouin, L. Auguardi, S. Tyagarajan.

Chexbres meeting on "Inhibitory Synapses" *(February-27 to March 1st, Chexbres, Switzerland.)* Poster presentation: ***Molecular Mechanism of Ischemia- Induced Changes at GABAergic Synapse.*** Z. S. Thirouin, R. Gill, L. Auguardi, A. Mc Kinney, S. Tyagarajan.

Gordon Research Seminar (June 15-16, Les Diablerets, Switzerland**) on "Inhibition in the CNS".** Poster presentation**:** ***Molecular Mechanism of BDNF- Induced Changes at GABAergic Synapse***. Z. S. Thirouin, R. Gill, L. Auguardi, A. Mc Kinney, S. K. Tyagarajan.:

Gordon Research Conferences (June 16-21, Les Diablerets, Switzerland**) on "Inhibition in the CNS",** Poster presentation**:** ***Molecular Mechanism of BDNF- Induced Changes at GABAergic Synapses***. Z. S.Thirouin, R. Gill, L. Auguardi, A. Mc Kinney, S.Tyagarajan.

Pharmocological Poster Day organized by UZH and ETH universities of Zurich (August 20th)**.** Poser presentation: ***Molecular Mechanism of BDNF- Induced Changes at GABAergic Synapse***. Z. S.Thirouin, R. Gill, L.Auguardi, A.Mc Kinney, S.Tyagarajan.:

SFN meeting in San Diego; Co-author on poster**:** ***BDNF Regulates Synapse Maintenance in Hippocampal CA1 Pyramidal Neurons After Oxygen-Glucose Deprivation*.** R.Gill, Z.Thirouin, S.K Tyagarajan, R. A. McKinney.

2015:

Annual meeting of the Swiss Society of Neurosciences *(January 24th, Fribourg, Switzerland)* Poster presentation: ***Molecular Mechanism of BDNF-Induced plasticity changes at GABAergic Synapse*** Z.S. Thirouin, S. Früh, J.-M.-Fritschy, S. K. Tyagarajan.

ZNZ Symposium (*September 11, Zurich, Switzerland*) Poster presentation***: BDNF signaling regulates PIAS-3 via two distinct mechanisms to modulate gephyrin scaffolding at GABAergic synapses***. Z. S. Thirouin, R. Gill, S. Früh, Jean-Marc-Fritschy, R.A Mckinney. S. K. Tyagarajan.

SFN meeting in Chicago; Co-author on poster**:** ***BDNF "highjacks" the SUMO pathway to induce synaptic GABAergic plasticity after ischemia*.** Z. S. Thirouin, R. Gil, R. A. McKinney, S. K Tyagarajan.

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